Molecular and Immunogenic Analysis of Jembrana Disease Virus Tat

This thesis is presented for the degree of
Doctor of Philosophy at Murdoch University

by

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DVM

2006

Declaration

I declare that this is my own account of my research and contains work that has not previously been submitted for a degree at any tertiary educational institution.

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Surachmi Setiyaningsih

Abstract

Jembrana disease is an acute and severe disease of Bali cattle (*Bos javanicus*) endemic in Indonesia that is caused by a bovine lentivirus designated *Jembrana disease virus* (JDV). Previous studies have demonstrated that it is possible to induce a protective immunity against the disease by immunisation with a crude whole virus vaccine prepared from the tissues of infected cattle. This vaccine has been demonstrated to ameliorate the clinical signs of disease resulting from exposure to virus infection but a safer vaccine amenable to commercial production techniques is required.

JDV, like all lentiviruses, encodes a transcriptional *trans*-activator Tat protein that is encoded from one or both of two exons of the *tat* gene. Tat is particularly essential for virus replication and it was hypothesised that the induction of an immune response in cattle against JDV Tat may effect protection against virus infection. Investigations were therefore conducted on JDV Tat to provide basic information on the protein that would enable it to be further investigated as a potential immunogen for incorporation into vaccines for the control of Jembrana disease.

Analysis of *tat* transcripts obtained from tissues of cattle infected with three strains of JDV suggested that, during the acute clinical disease, Tat produced at this stage of the disease process was translated from the first coding exon only. Nucleotide variation in this exon, which would have translated into amino acid variations in the Tat protein, was evident especially between strains from geographically different regions of Indonesia. There was; however, conservation of the essential functional domains of cysteine-rich, core and basic regions, which suggested immunity to a single Tat protein might protect against infection by heterologous strains. Subsequent studies on Tat reported in the thesis therefore concentrated on the protein encoded by *tat* exon 1 of a single strain of JDV.

The exon 1 of *tat* was cloned into the pGEX vector and recombinant Tat expressed in *Escherichia coli*. Methods for the purification of the expressed protein were developed. Immunogenicity of the recombinant protein was initially demonstrated by inoculation of the protein into a sheep which developed a high titred specific antibody response. Antibodies induced by this recombinant protein recognised native Tat proteins produced by three JDV strains in Bali cattle and provided a valuable reagent for the subsequent detection of Tat *in vitro* and *in vivo*.

Aspects of the antibody response to Tat were determined in cattle that had been infected naturally or experimentally with JDV, and compared with the levels of antibody to the immunodominant capsid protein. Tat antibodies were detected in 23 % of 128 Bali cattle from Jembrana disease-endemic areas of Indonesia; in all these cattle, evidence of previous virus infection had been demonstrated by detection of antibody to the JDV capsid protein by Western blot analysis. In cattle experimentally infected with JDV, low levels of serum antibody to Tat were detected by Western blot in the first month post-infection but the levels of antibody then decreased; levels of antibody to the JDV capsid protein increased over the 6-month observation period following infection. The detection of Tat-antibody soon after the acute clinical disease suggested that this protein is secreted extracellularly during JDV infection in cattle. In contrast to the antibody response to Tat in JDV-infected cattle, an apparently greater antibody response to Tat was induced by injection of recombinant Tat in Bali cattle. The strong antibody response resulting from inoculation of the recombinant Tat and low levels of Tat antibody in animals that had been naturally or experimentally infected with virus suggested there might be a conformational difference in the recombinant and native Tat protein and that the native protein was a poor immunogen, or that the levels of Tat in infected cattle were too low to induce a strong antibody response.

As an alternative means of inducing an immune response to JDV Tat, perhaps one associated with a greater cell-mediated rather than an antibody response, a candidate *tat* DNA vaccine was produced by insertion of *tat* exon 1 into a DNA vaccine vector. Transfection of this naked DNA plasmid into mammalian cells induced the expression of a functional Tat protein which maintained antigenicity. The results suggested this construct merits further animal studies attempting to induce a protective immune response against Jembrana disease in cattle. A method of assaying the *trans*-acting function of Tat was also developed which will have application for quality control procedures for large-scale production of *tat* DNA vaccine.

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Acknowledgments

I would like to express my gratefulness to my supervisor, Prof. Graham Wilcox, for his focused guidance and motivation throughout my PhD candidature. His constructive suggestions and careful review of my thesis have enabled me to finish my work. It has been a great opportunity for me to be a member of his laboratory and work under his supervision.

I would like to thank all the great people in Veterinary Virology group, past and present, for invaluable support, friendship and challenges, and for creating a great atmosphere. Special thanks to Moira, Meredith, Will, Steve, Evan and Muhammad for stimulating discussion and technical advice, and big fellows Drew and Mark for encouragement and support during hard situation. I would also like to thanks to the Bali Cattle Disease Investigation Unit (Denpasar, Indonesia) for the precious samples and animal experimentation.

General thanks to State Agricultural and Biotechnology Centre (SABC) for excellent facilities and services. My personal thanks to Cecily for her inspiring "Paragraphs on Paper", and Rini for the great friendship and gastronomic skill.

I am forever grateful to my husband and my children for their unconditional support and understanding throughout my study. And most of all, my eternal grateful to my mum for her continuous and loving support.

Without those wonderful people this work would not have been possible.

To my children:

Sekar, Puspa, Wira and Surya

Abbreviations

aa	Amino acid
Ab	Antibody
AIDS	Acquired immune deficiency syndrome
ALV	*Avian leucosis virus*
ANGIS	Australian National Genomic Information Service
APC	Antigen presenting cell
APS	Ammonium persulfate
ARC	AIDS-related complex
BIV	*Bovine immunodeficiency virus*
BLV	*Bovine leukaemia virus*
bp	Base pair
BSV	*Bovine spumavirus*
CA	Capsid protein
CAEV	*Caprine arthritis-encephalitis virus*
CCR5	C-C (beta) chemokine receptor 5
CDK9	Cyclin Dependent Kinase 9
cDNA	Complementary DNA
CMV	*Cytomegalovirus*
CTD	C-Terminal Domain of the major subunit of RNA Polymerase II
CTL	Cytotoxic T lymphocyte
CXCR4	C-X-C (alpha) chemokine receptor 4
dATP	Deoxyadenosine triphosphate
DC	Dendritic cell
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
dNTPs	Deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP)
dsDNA	Double-stranded DNA
dTTP	Deoxythymidine triphosphate
ECL	Enhanced Chemoluminiscense
E. coli	*Eschericia coli*
EDTA	Ethylenediamine tetra acetic acid

EIAV	*Equine infectious anaemia virus*
ER	Endoplasmic reticulum
ESE	Exon splicing enhancer sequence
ESS	Exon splicing silencer sequence
FBL	Foetal bovine lung cells
FBS	Foetal bovine serum
FITC	Fluorescein isothiocynate
FIV	*Feline immunodeficiency virus*
Gag	Group-specific antigen
GCG	Genetics Computer Group
gDNA	Genomic DNA
gp	Glycoprotein
GSH	Reduced glutathione
GST	Glutathione-S-transferase
HAART	Highly active antiretroviral therapy
HIV	*Human immunodeficiency virus*
HRP	Horseradish peroxidase
HTLV	Human T-lymphotropic virus
IFA	Immunofluorescence antibody assay
IL	Interleukin
IN	Integrase
IPTG	Isopropyl-β-thiogalactopyranoside
ISH	*In situ* hybridisation assay
JDV	*Jembrana disease virus*
KS	Kaposi's sarcoma
LB	Luria-Bertani medium
LTR	Long terminal repeat
MA	Matrix protein
mAb	Monoclonal antibody
MDBK	Madin-Darby bovine kidney cells
MHC	Major histocompatibility
mRNA	Messenger RNA
(M)MuLV	(*Moloney*) *murine leukemia virus*
MVV	*Maedi visna virus*
NC	Nucleoprotein
Nef	Negative factor
Ni-NTA	Nickel-nitrilotriacetic acid

ORF	Open reading frame
p.i.	Post-inoculation/infection
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline, Primer binding site
PCR	Polymerase chain reaction
PIC	Pre-integration complex
PLV	*Puma lentivirus*
PMSF	Phenyl Methyl Sulfonyl Fluoride
PR	Protease
PTEF	Positive Transcription Elongation Factor
Rev	Regulator of expression of virion proteins
RGD	The amino acid triplet arginine-glycine-aspartic acid sequence
RNA	Ribonucleic acid
RNAP II	RNA Polymerase II
Rnase	Ribonuclease
RRE	Rev-responsive element
RSV	*Rous sarcoma virus*
RT	Reverse transcriptase
SA	Splice acceptor
SD	Splice donor
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SIV	*Simian immunodeficiency virus*
SIVagm	*Simian immunodeficiency virus* African green monkey
SIVcpz	*Simian immunodeficiency virus* chimpanzee
SIVmnd	*Simian immunodeficiency virus* mandrill
SIVsmm	*Simian immunodeficiency virus* sooty mangabey monkey
SIVsyk	*Simian immunodeficiency virus* Skye's monkey
SRLV	Small-ruminant lentiviruses
SRV	*Simian retrovirus*
ssRNA	Single-stranded DNA
ssRNA	Single-stranded RNA
SU	Surface unit glycoprotein
TAR	*Trans*-activating response element
Tat	*Trans*-activator of transcription protein
TBS	Tris-buffered saline
TE	Tris-EDTA buffer

TEMED	N,N,N',N'-tetra methyl ethylene diamine
T_m	Melting temperature of dsDNA
TM	Transmembrane glycoprotein
Tris	Tris(hydroxymethyl)aminomethane
WB	Western blotting
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

List of Units

°C	degrees Celsius
μg	micrograms
μL	microlitre
μM	micromolar
ρmol	picomoles
bp	base pairs
d	days
g	grams
g	times gravity
h	hours
ID_{50}	50 % infectious dose
kb	kilobases
kDa	kiloDalton
M	molar
mA	milliAmperes
mg	milligrams
min	minutes
mL	millilitre
mM	millimolar
ng	nanograms
nm	nanometre
OD	optical density
rpm	revolutions per minute
s	seconds
U	Units of enzyme activity
V	volts
v/v	volume per volume
w/v	weight per volume

Chapter 1

General Introduction

Jembrana disease virus (JDV) is a member of the *Lentivirus* genus of the family *Retroviridae*, which causes an acute and severe infection in Bali cattle that is endemic in parts of Indonesia. A high plasma viral load has been shown to coincide with the acute disease syndrome that occurs soon after infection. An inactivated virus vaccine prepared from infected spleen extract has been produced and is used within Indonesia for the control of the disease (Hartaningsih *et al.*, 2001) and shown to ameliorate the acute disease process. Although this vaccine has many disadvantages its use has demonstrated that vaccination is a feasible method of control of the disease. An alternative safer and higher quality vaccine is required, and the studies reported in this thesis were conducted in support of this objective.

In addition to the *gag*, *pol*, and *env* genes characteristic of retroviruses, the lentiviruses encode several accessory and regulatory genes. The *tat* gene encodes a regulatory protein Tat, a potent viral transcriptional *trans*-activator that is absolutely required for virus replication. Because of its important biological and pathological roles in *Human immunodeficiency virus* (HIV) infection (Huigen *et al.*, 2004), the Tat protein has been considered as an attractive candidate protein for the development of *Human immunodeficiency virus 1* (HIV-1) vaccines. The principal aim of the investigations reported in this thesis was to investigate the potential of using JDV Tat as an immunogen for the control of Jembrana disease. This required preliminary *in vitro* investigation of the transcription and translation of *tat* and the construction of appropriate recombinant Tat proteins and naked *tat* DNA constructs that might be trialed as potential vaccines.

Preliminary to this investigation, a review of the literature relating to the lentiviruses in general and particularly to bovine lentiviruses and their associated

diseases was conducted and is provided in Chapter 2. This provided background to the investigation and the relevance and direction of the research. Features of the lentivirus genome, including the various gene products are described in the review, with particular emphasis on the role of *tat* and the encoded Tat in the pathogenesis of disease. The potential use of Tat as a vaccine component is discussed with special reference to investigations that have been conducted with HIV-1. These were reviewed as they provide an insight into the potential for induction of an immune response to JDV Tat as a means of controlling Jembrana disease in cattle.

The mechanism of *tat* expression in infected animals was considered important in determining the probable characteristics of the expressed Tat protein, and in understanding the pathogenesis of Jembrana disease. Investigations of *tat* transcription in animals with acute Jembrana disease were conducted and are reported in Chapter 3. These studies included an investigation of possible *tat* sequence variation in 3 different JDV strains as this was considered important in defining possible antigenic variation in Tat.

An antibody response in infected cattle has been demonstrated using whole virus as an antigen in serological assays (Hartaningsih *et al.*, 1994); the antibodies detected were considered to be primarily those against the immunodominant capsid protein of JDV. Evidence for the occurrence of antibodies in JDV-infected cattle that are reactive to Tat has not been reported. To facilitate such investigations into the antibody response to Tat, and to provide a potential Tat immunogen for induction of a protective immunity against Jembrana disease in cattle, a bacterially expressed recombinant Tat fused to glutathione S-transferase was produced. These studies on the immunogenicity and antigenicity of JDV Tat are reported in Chapter 4.

DNA vaccines are thought to result in more efficient antigen presentation to the immune system and a greater T-cell-mediated (rather than antibody-mediated) immune response than protein-based vaccines. Hence a *tat* DNA construct was developed and investigations of its expression and function *in vitro* were

conducted. An assay to determine the functional activity of expressed Tat proteins was developed to facilitate these studies. These preliminary studies on the development of a JDV *tat* DNA vaccine candidate are presented in Chapter 5

A general discussion of the investigations conducted and the results obtained in Chapter 3, 4 and 5 is provided in Chapter 6.

Chapter 2

Review of the Literature

This chapter reviews the characteristics of retroviruses with emphasis on lentiviruses, particularly the role of the transcriptional *trans*-activator protein (Tat) in lentivirus replication and pathogenesis of lentivirus-associated disease. In addition, the literature describing the immune response to Tat and the use of Tat as a vaccine to control lentivirus infection are discussed with special reference to the *Human immunodeficiency virus* type-1 (HIV-1).

The family *Retroviridae*

Retroviruses comprise a large and diverse group of ribonucleic acid (RNA)-containing viruses that infect primarily vertebrates (Coffin, 1992a). The hallmark of the family is that replication involves an essential reverse transcription of the virus RNA into a linear double-stranded (ds) DNA and the subsequent integration of this dsDNA into the genome of the cell (Baltimore, 1970; Temin & Mizutani, 1970; Temin, 1976).

Typical retroviruses are enveloped particles ranging from 80 to 130 nm in diameter with an internal conical or spherical shaped core or capsid (CA) that is surrounded by matrix (MA) protein. The core contains the viral RNA genome stabilised by a nucleocapsid (NC) protein, cellular tRNA primer and several viral encoded enzymes including reverse transcriptase (RT), integrase (IN) and protease (PR) (Vogt, 1997). The envelope is a lipid bilayered membrane that is derived during maturation of the virus from the host cell membrane during a budding process, requiring modification of the cell membrane by insertion of two viral-coded glycoproteins, a surface (SU) glycoprotein and a *trans*-membrane (TM) glycoprotein.

The retrovirus genome consists of two identical linear, positive-sense, single-stranded (ss) RNA molecules non-covalently joined as a dimer at the 5` end, that are capped and polyadenylated like cellular mRNA (Berkowitz *et al.*, 1996). The genome of prototypical retroviruses contains the major genes, *gag*, *pro*, *pol* and *env* in that order, which encode for precursor polyproteins that are subsequently cleaved into the various structural and non-structural proteins of the virus. In some retroviruses *pro* is part of either *gag* or *pol*. Flanking the coding genes are untranslated redundant (R) sequences at both termini along with adjacent unique (U) sequences U5 and U3, positioned at the 5' and 3' ends of the RNA genome, respectively. In the proviral (DNA) form the flanking regions are known as long terminal repeats (LTR), each composed of U3-R-U5 (Vogt, 1997).

Retroviruses were originally classified into four morphologically distinct forms depending on the features of the core detected by electron microscopy, and were designated as types A, B, C and D (Coffin, 1992a; Luciw & Lesing, 1992). Viruses were also grouped according to differences in pathogenicity into three subfamilies: Oncovirinae, Lentivirinae and Spumavirinae (Matthews, 1982). However, more recent analysis of the genome has resulted in reclassification of the viruses into seven genera: *Alpharetrovirus*, *Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, *Spumavirus* and *Lentivirus* (van Regenmortel *et al.*, 2000).

The first five genera are often referred to as oncogenic retroviruses as they are associated with a variety of leukaemias and sarcomas in a wide variety of animal species. The Spumaviruses, also known as "foamy" viruses, are highly cytopathic inducing marked syncytium formation and vacuolation in many types of cells *in vitro* and although they are known to cause persistent infection of several animal species they have not been definitively linked to any specific disease (Meiering & Maxine L. Linial, 2001; Rosenberg & Jolicoeur, 1997). The several species of the genus Lentivirus cause a variety of syndromes including immunodeficiencies and neurological disorders in primates and feline species, chronic pneumonia, arthritis and encephalitis in goats and sheep, and anaemia in equine species (Desrosier, 2001; Rosenberg & Jolicoeur, 1997).

Characteristic of the genus *Lentivirus*

The lentiviruses infect a diverse array of mammalian hosts, and can be categorised into five different groups accordingly: Primate lentivirus group (*Human immunodeficiency virus* [HIV-1 and -2] and *Simian immunodeficiency virus* [SIV]), Feline lentivirus group (*Feline immunodeficiency virus* [FIV] and *Puma lentivirus* [PLV]), Equine lentivirus group (*Equine infectious anaemia virus* [EIAV]), Bovine lentivirus group (*Bovine immunodeficiency virus* [BIV] and *Jembrana disease virus* [JDV]), Ovine/caprine lentivirus group (*Maedi-visna virus* [MVV] and *Caprine arthritis-encephalitis virus* [CAEV]). The relationship between lentiviruses has been established molecularly by comparing the conserved *pol* gene particularly the RT coding region (Li *et al.*, 1995b; Xiong & Eickbush, 1990). Figure 2.1 shows a phylogenetic relationship of several lentiviruses based on the analysis of their complete *pol* genes encompassing PR, RT and IN coding regions.

Lentiviruses have a distinctive morphology. Assembly of the lentivirus particles and budding occurs simultaneously at the plasma membrane of the infected host cell, similar to type C virion, leading to immature virions with hollow conical cores. The mature virion has an envelope with visible projections, and the capsid is arranged into a distinctive truncated cone-shaped rather than central and spherical as a C-type (Desrosier, 2001). In addition, lentiviruses differ from the prototypic simple retroviruses in that their genome and replication cycle is more complex (Vogt, 1997).

Genomic organisation and gene products of lentiviruses

Lentiviruses have a similar genomic organisation that differentiates from that of other genera, but individual lentiviruses differ in the degree of complexity of the genome. Illustrated schematically in Figure 2.2, the genome contains the three major open reading frames (ORFs) common to all retroviruses; *gag*, *pol* and *env*; *pro* is in-frame and contiguous with *pol*. In addition, lentiviruses carry a variable number of distinctive regulatory and accessory genes, most of which are found

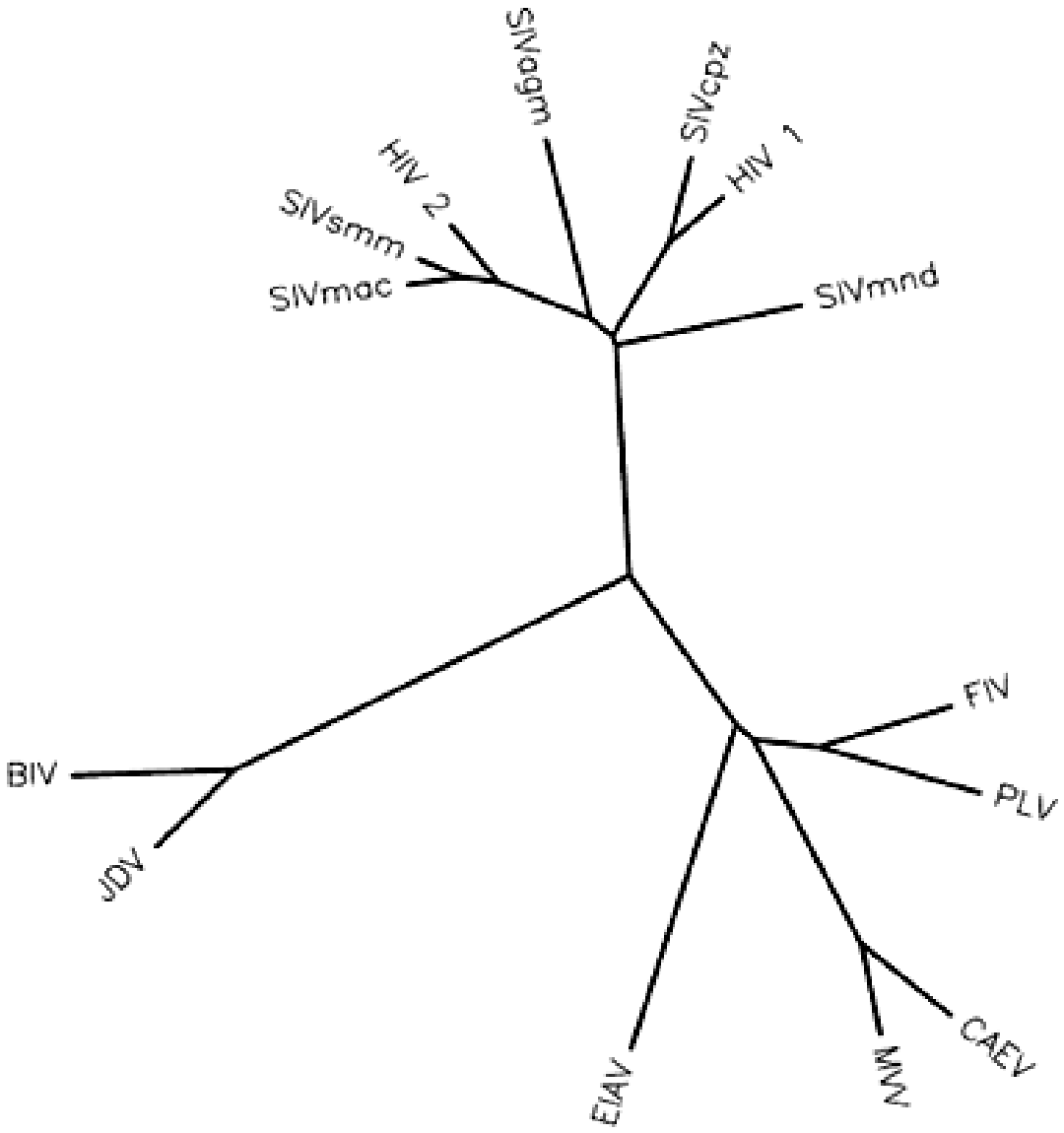

Figure 2.1. Phylogenetic tree of lentiviruses. The genetic relationship of several lentiviruses was analysed based on complete *pol* gene sequences downloaded from GeneBank. The sequences were aligned and the tree was built using several programs accessible at ANGIS. BIV, *Bovine immunodeficiency virus*; JDV, *Jembrana disease virus*; EIAV, *Equine infectious anaemia virus*; MVV, *Maedi-visna virus*; CAEV, *Caprine arthritis encephalitis virus*; FIV, *Feline immunodeficiency virus*; PLV, *Puma lentivirus*; HIV-1, *Human immunodeficiency virus 1*; HIV-2, *Human immunodeficiency virus 2*; SIVagm, *Simian immunodeficiency virus* (African green monkey isolate); SIVcpz, SIV (chimpanzee isolate); SIVmac, SIV (macaque isolate); SIVmnd, SIV (mandrill isolate); SIVsmm, SIV (sooty mangabey monkey isolate).

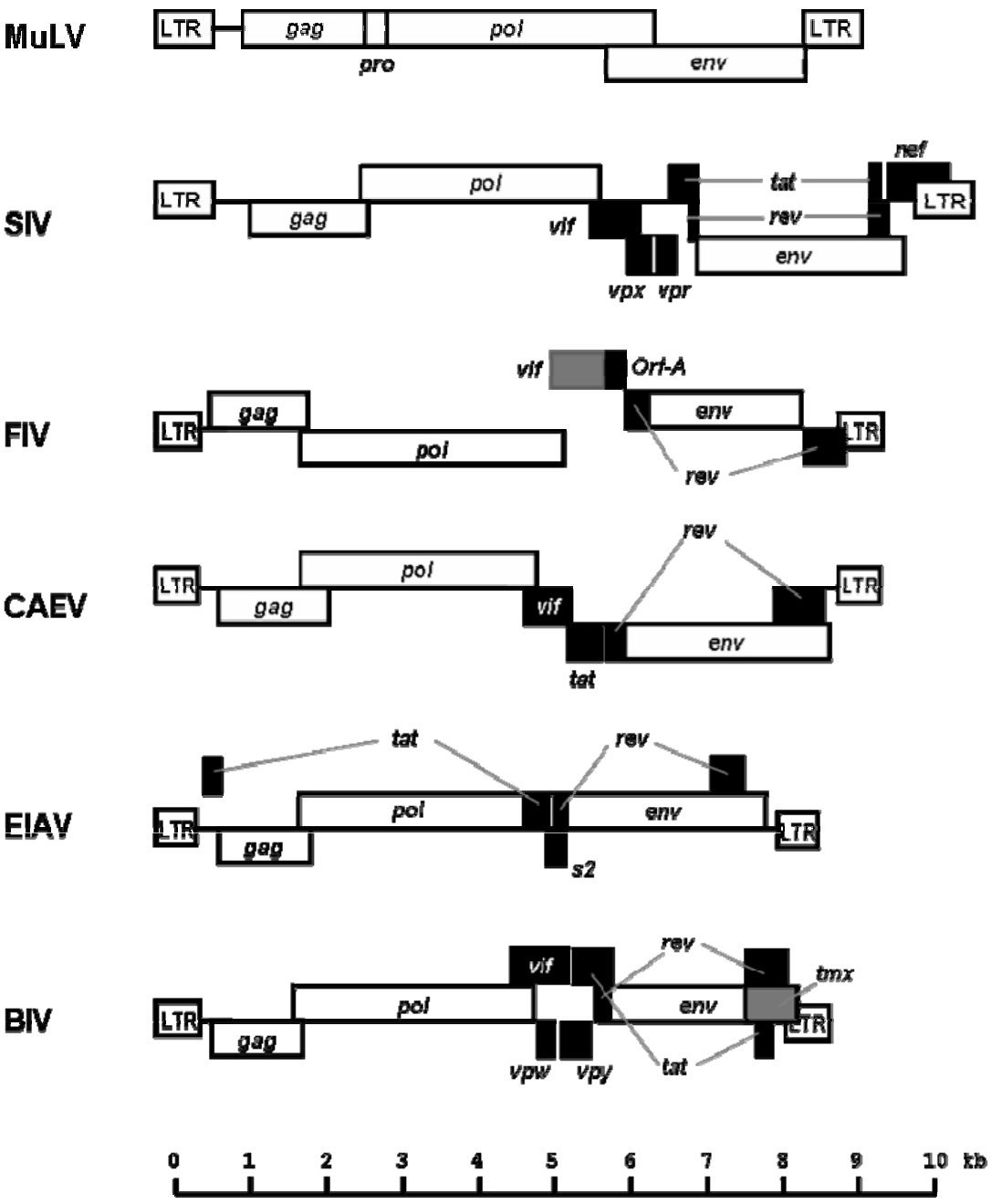

Figure 2.2. Genomic organisation of lentiviruses. Lentiviruses are complex retroviruses with accessory and regulatory genes in addition to *gag*, *pol*, *pro* and *env* that form simple retroviruses such as murine leukemia virus (MuLV). The *pro* is not indicated as it is in frame with the *pol* gene.

overlapping the central region between the end of *pol* and the beginning of *env*; and in several viruses also post-*env* (Vogt, 1997).

The most complex of the lentiviruses, HIV-1, contains *tat* and *rev* genes encoding viral proteins with regulatory functions and genes *vif*, *vpu*, *vpr*, and nef with accessory/auxiliary functions (Emerman & Malim, 1998; Frankel & Young, 1998; Freed & Martin, 2001; Piguet & Trono, 1999; Pollard & Malim, 1998). The organisation of these regulatory and accessory genes of HIV-1 with the respective encoded proteins in the virion structure is depicted in Figure 2.3.

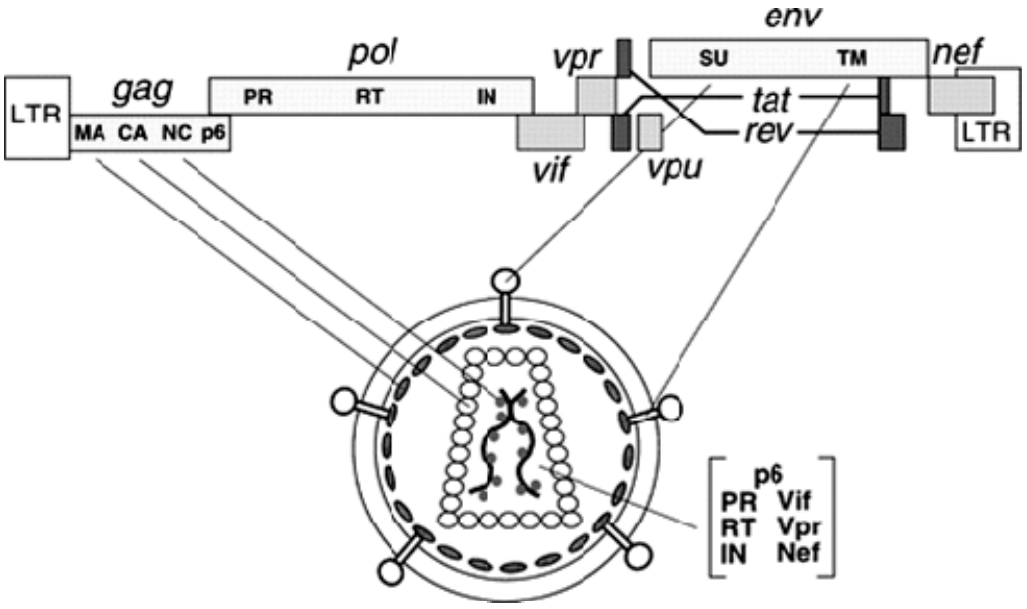

Figure 2.3. Genomic and structural organisation of HIV-1. Positions of the major viral proteins, the lipid bilayered membrane, and the diploid genomic RNA are indicated. Reproduced from Frankel & Young (1998).

The *gag* ORF encodes a 55 kilodalton (kDa) Gag (group-specific antigen) precursor polyprotein ($Pr55^{Gag}$) that is proteolytically cleaved into internal structural matrix protein of 17 kDa, the capsid protein of 24 kDa, the nucleocapsid protein of 7 kDa and the p6 (Swanstrom & Wills, 1997). The MA protein is myristoylated at the N-terminus, which mediates the binding of the $Pr55^{Gag}$ to the

plasma membrane of the host cell giving it a key role in the assembly of infective virus (Freed, 1998). Once cleaved, the MA protein is located in the matrix between the capsid and viral envelope. The CA protein is the major structural protein of the virus; it forms the protein coat surrounding the genome and is an immunodominant viral protein (Coffin, 1992b). The NC protein has an affinity for and is associated with the viral RNA genome and is required for packaging RNA into the virion. The carboxyl-terminal p6 domain is required for virion release (Gottlinger *et al.*, 1991; Huang *et al.*, 1995) and for efficient incorporation of the viral accessory protein Vpr into virions (Kondo & Gottlinger, 1996).

The *pol* region directs the synthesis of three virion-associated enzymatic proteins, the protease, reverse transcriptase and integrase enzymes, and is translated as an extended Gag-Pol polyprotein ($Pr160^{Gag\text{-}Pol}$) following a ribosomal frame-shift event (Vogt, 1997). The PR, which is active in a homodimeric form (Navia *et al.*, 1989), is responsible for all the proteolytic cleavage of the precursor polyproteins, generating the mature Gag and Pol proteins during assembly and maturation of the viral particles (Mervis *et al.*, 1988). The RT has both RNA-dependent DNA polymerase and RNase H activities that are required to convert the ss viral RNA genome into dsDNA upon entry into host cells. RT binds the tRNA primer (essential for initiation of DNA synthesis shortly after infection), and a separate domain on this protein functions as a ribonuclease specific for RNA-DNA hybrids (RNase H). The biologically active form of HIV-1 RT is a heterodimer composed of a 51-kDa and a 66-kDa subunit (p51/p66). RT is assembled into virions as part of a precursor Gag-Pol polyprotein which is generated during proteolytic cleavage by the *pol*-encoded protease (PR) (Katz & Skalka, 1994; Oroszlan & Luftig, 1990; Telesnitsky & Goff, 1997). The p66 subunit contains the DNA polymerase and RNase H domains, while the p51 subunit lacks the RNase H domain (Hizi *et al.*, 1988; Prasad & Goff, 1989). Each virion contains 10–20 molecules of RT which are loosely associated with the nucleoprotein core. IN is a separate protein derived from the carboxyl terminus of the Gag-Pol polyprotein that catalysis the irreversible integration of the viral cDNA, generated by reverse transcription of the viral RNA genome, into host cell DNA (Coffin, 1979; Coffin, 1992b).

The *env* ORF encodes a polyprotein, gp160, which is post-translationally modified in the endoplasmic reticulum via cleavage and glycosylation events mediated by cellular enzymes (Hallenberger *et al.*, 1997) to produce two glycoprotein subunits, a surface (SU) gp120 molecule and *trans*-membrane (TM) gp41 (Coffin, 1992b). The SU is located on the external surface of the viral membrane and it is responsible for the binding of the virus to host cell receptors. The TM is embedded into the lipid bilayer envelope and anchors the SU domain to the membrane and facilitates the fusion of viral and cell membranes during entry. These envelope glycoproteins are noncovalently linked as heterodimers that associate further into trimers (Weiss *et al.*, 1990) to form the short spikes on the surface of the virion. The glycoproteins are the primary targets of the neutralising antibody response and are the determinants of tropism and virulence (Coffin, 1992a).

The accessory proteins Vif, Vpr and Vpu appear to orchestrate a variety of functions. They are not required for viral growth *in vitro* but are essential for viral replication and pathogenesis *in vivo* (Trono, 1995). For example, infection of rhesus monkeys with a *vpr*- and *vpx*-defective SIV produced a low virus burden and they did not develop immunodeficiency disease (Gibbs *et al.*, 1995). The viral infectivity factor (Vif) of HIV-1 is encoded by an essential accessory gene that is conserved in all lentiviruses with the exception of EIAV (Oberste & Gonda, 1992). Vif enhances the infectivity of virus particles produced in non-permissive cells and the stability of viral DNA, and it also may play a role in virion assembly (Simon & Malim, 1996). Vpr (viral protein R) assists in the transport of viral components into the nucleus promoting productive infection in non-dividing cells and it may induce an arrest of cell cycle progression in the G2 phase preventing the subsequent events of mitosis and division of HIV-1-infected cells, which provides a replication advantage for the virus (Cohen, 1996; Frankel & Young, 1998). Both Vif and Vpr are incorporated into the virion particles. The Vpu is a small integral membrane protein that promotes degradation of intracellular CD4 molecules, facilitating transfer of newly synthesised Env molecules to the cell

membrane for virion assembly, and it can also down-regulate MHC class proteins and stimulate virion release (Frankel & Young, 1998).

Nef (negative factor) is a protein unique to the primate lentiviruses that is incorporated into the virion core. Nef is expressed rapidly and abundantly following infection *in vitro* and *in vivo*. Nef is a major virulence factor *in vivo*: it down-regulates the CD4 receptor by binding to the cell surface, as well as MHC class I levels, which allows HIV-1 infected cells to escape from cytotoxic T-lymphocyte-mediated lysis (Collins *et al.*, 1998); extracellular Nef may also stimulate latently infected cells into productive HIV-1 infection.

The regulatory genes *tat* and *rev* each consist of two exons generated by double or multiple splicing of viral RNA. Rev is a small, 13-kDa sequence-specific RNA-binding phosphoprotein that shuttles between the nucleus and the cytoplasm to promote transport of unspliced and singly spliced viral RNA from the nucleus to the cytoplasm. It binds to the *rev*-responsive element (RRE), a *cis*-acting sequence within the *env* gene (Felber *et al.*, 1990), permitting expression of viral structural proteins. Tat activates the viral promoter at the 5' LTR, leading to more efficient transcription of the proviral genome. The Tat protein recruits cellular proteins and binds to the *trans*-acting response element (TAR), a *cis*-acting sequence in the LTR, to upregulate transcription (Arya *et al.*, 1985). The action of Tat thus forms a positive-feedback mechanism that greatly accelerates viral transcription once a threshold level has been reached. Interaction of Tat with TAR is indispensable for viral replication. The structure and functions of Tat was reviewed in more detail in separate sections.

Bovine immunodeficiency virus

In terms of genome organisation, BIV is the most complex non-primate lentivirus (Garvey *et al.*, 1990; Gonda *et al.*, 1994; Oberste *et al.*, 1993). Besides genes encoding obligate retrovirus Gag, Pol, and Env polyproteins, BIV has genes *tat, rev, vif, vpw* and *vpy* located in the central region of the genome, and *tmx* located in the 3' end of *env* (Figure 2.2) that encode six accessory proteins, designated

Tat, Rev, Vif, Vpw, Vpy, and Tmx, respectively. These proteins play a critical role in the viral replication cycle and contribute significantly to the pathogenesis of BIV.

BIV resembles HIV in its structural, genomic, antigenic, and biological characteristics. The predicted and/or known gene products of the accessory genes *vif* and *tat*, as well as those of the structural genes *gag*, *pol* and *env* have some sequence similarity to their counterparts in HIV-1 (Battles *et al.*, 1992). Characterisation of the *gag* gene and the encoded 53 kDa precursor protein ($Pr53^{Gag}$) was reported by (Battles *et al.*, 1992; Tobin *et al.*, 1994). The major BIV 53 kDa precursor protein ($Pr53^{Gag}$) was cleaved into MA, CA, and NC gag proteins of p16, p26, and p13, respectively. In addition to BIV Pr53gag, the major gag precursor, two other gag-related precursors of 170 and 49 kDa were identified, as were several alternative gag cleavage products. Mono-specific antisera to HIV-1 CA (p24) and NC (p7) proteins cross-reacted with analogous BIV proteins which aided in the identification (Atkinson *et al.*, 1992; Battles *et al.*, 1992).

There are also significant differences in the genomes of the BIV and HIV. In BIV, the genome locations and other conserved traits in the predicted products of *rev* and *vpw* and *vpy* ORFs suggest that they are probably analogous to *rev* and *vpr, vpu,* and *vpx* genes, respectively, in the primate lentiviruses. The function of the BIV *tmx* gene is not known, but it resides in the 3' end of the genome in a position analogous to the *nef* gene of primate lentiviruses.

Replication cycle of lentiviruses

Unique among animal viruses, retroviral replication proceeds through an obligate recombination step with host cell DNA and the major steps are depicted schematically in Figure 2.4. The early phase involves attachment of viruses via surface glycoproteins to the main cellular receptor CD4 followed by interaction with a co-receptor and subsequent fusion with the host cell. The viral ssRNA

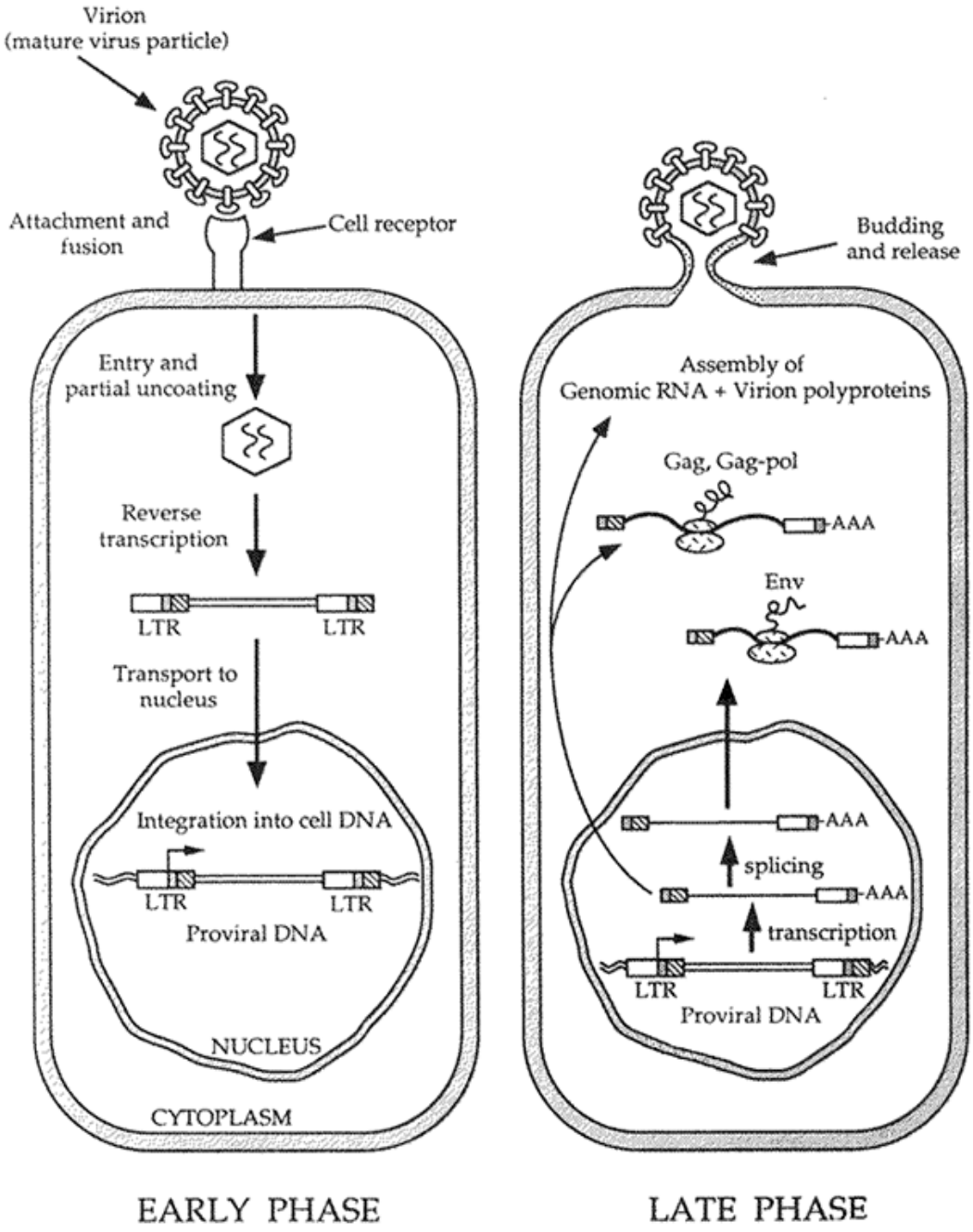

Figure 2.4. Major steps in the replication cycle of a typical retrovirus. Reproduced from Flügel (1993)

genome is subsequently converted into dsDNA by the viral enzyme reverse transcriptase and transported into the nucleus where the viral integrase mediates the integration of the viral DNA into the host genome. In the late phase proviral DNA is transcribed by cellular enzymes to generate the spliced and unspliced viral mRNAs that are then translocated to the cytoplasm for translation. The viral RNA and structural proteins are assembled into the immature virion core on the plasma membrane where it buds to form free virus and becomes mature.

Attachment of virus to host cells and uncoating

The attachment of the virion to the host cell receptor is the first step in the entire genetic delivery mediated by specific interactions between the envelope glycoprotein on the virion and one or more surface receptor molecules on the target cell. This interaction is specific and selective for particular cells and therefore particular tissue types, and is associated with host cell specificity of the different species (Clapham & McKnight, 2002; Overbaugh *et al.*, 2001).

Attachment of HIV-1 to cells is mediated by the virion SU binding to CD4 molecules, the major receptor on the surfaces of T-helper lymphocytes (T4-cells or CD4+ cells) and other antigen presenting cells such as macrophages, monocytes and dendritic cells. This binding induces a conformational change that orientates the chemokine receptor-binding domains of SU into close proximity with the host cell chemokine receptors (CCR5 receptors on macrophages, CXCR4 receptors on T4-lymphocytes and CCR3 receptors on microglia cells of the brain). In a subsequent conformational change, a previously buried portion of the TM protein gp41 is exposed, enabling the viral envelope to fuse with the host cell membrane (Sherman & Greene, 2002). The fusion results in the release of the viral core molecule, containing the viral genome and enzymes, into the cytoplasm. Alternatively, the virus might enter via endocytosis after which the envelope fuses with the endocytic vesicle releasing the genome-containing core into the cytoplasm.

Reverse transcription of viral genome and its integration into cellular genome

Upon entry into the cytoplasm, retroviruses are uncoated. In the cytoplasm, the viral RNA is converted into dsDNA by the viral RT; the viral nucleic acids remain associated with a number of viral proteins throughout these steps. The process of reverse transcription is a highly conserved function within the family Retroviridae, which occurs within the nucleoprotein complex and is initiated by the binding of cellular tRNA (tRNALys) to the primer binding site (PBS) located downstream to the 5' LTR of the viral genomic plus-stranded RNA (Gerdts *et al.*, 1997). The minus-stranded DNA complementary to the 5' U5 and R region is then synthesised and the complementary RNA template is partially degraded by the ribonuclease H (RNAse H) activity of the reverse transcriptase. The synthesised minus-stranded DNA is thereafter transferred to the 3' end of the RNA, where its R sequence hybridises with the plus-stranded 3' R sequence. The minus-stranded DNA is extended, and most of the plus-stranded RNA is digested except the polypurine tracts (PPT) that serve as a primer for the synthesis of the 3' part of the complementary strand of DNA (plus-stranded DNA). The PPT RNA sequences are then removed by RNAse H and a second strand transfer of the minus-stranded DNA allows the hybridisation of the PBS sequences of both DNA strands. This process provides free 3' ends for completion of the synthesis of both plus and minus strands of DNA, generating the ds linear DNA ready for integration. The viral DNA is contained in a protein-DNA complex known as the pre-integration complex (PIC) (Bowerman *et al.*, 1989; Miller *et al.*, 1997).

How the PIC enters the nucleus remains unclear (Depienne *et al.*, 2001; Devroe *et al.*, 2003). In the simple retroviruses, nuclear localisation of the PIC requires cell division (Lewis & Emerman, 1994; Miller *et al.*, 1990; Roe *et al.*, 1993), while for the lentiviruses, nuclear importation of PIC can occur even in non-dividing cells (Lewis *et al.*, 1992). Once inside the nucleus, the linear DNA serves as the immediate precursor for the formation of the integrated virus dsDNA or "provirus" (Brown, 1997). The integration reaction requires three discrete steps: assembly of a stable pre-integration complex at the termini of the viral DNA, and two sequential transesterification reactions. In the 3'-processing reaction,

endonucleolytic cleavage of the two 3' nucleotides at each DNA end generates 3'-hydroxyl group that provides the site for joining with the 5'-ends of the target host DNA in the strand transfer reaction. The product of integration is a gapped intermediate in which the non-joined 5'-viral DNA ends are flanked by short single-stranded gaps in the host DNA. Removal of mispaired nucleotides and gap repair are carried out by cellular enzymes (Engelman, 2003).

Once integrated, the HIV proviral DNA exists in either a latent or productive state, determined by genetic factors dependent on the virus strain, the type of cell infected and the production of specific host cell proteins. The majority of proviral DNA is integrated into the chromosome of active T4-lymphocytes. These generally comprise 93–95 % of infected cells and are productively infected. A small percentage of HIV-infected memory T4-lymphocytes persist in a resting stage due to a latent provirus. These, along with infected monocytes, macrophages and dendritic cells, provide reservoirs of HIV capable of escaping host defences and antiretroviral therapy (Blankson *et al.*, 2002; Zhang *et al.*, 1999).

Circular forms of viral DNA containing either one or two copies of the LTR can be found in the nucleus (Hindmarsh & Leis, 1999; Lobel *et al.*, 1989). These viral DNAs are generally believed to be transcriptionally silent remnants of dsDNA that fail to integrate (Sakai *et al.*, 1993). It has been reported; however, that in certain mutants defective in integrase function these are capable of limited viral gene expression including expression of Tat (Stevenson *et al.*, 1992; Wiskerchen & Muesing, 1995). Integrated provirus is essential for HIV-1 replication (Englund *et al.*, 1995; Goff, 1992; Whitcomb & Hughes, 1992) but during the asymptomatic phase the most prevalent form of HIV-1 DNA in resting and activated CD4+ T cells is a full-length, linear, unintegrated form that is not replication competent (Chun *et al.*, 1997).

Integrated proviral DNA maintained within host chromosomal DNA is an active template for the expression of retroviral gene products. The proviral DNA serves as a single expression unit, where a single primary mRNA is transcribed from the

5’ long terminal repeat (LTR) promoter, and is terminated at the 3’LTR, which also provides the polyadenylation signal for cellular RNA polymerase II (Goff, 2001).

Transcription of mRNA

Splicing plays a key role in the production of mRNA for the retroviral proteins and a summary of the events involved is shown in Figure 2.5. More than 30 different mRNA transcripts generated through alternative splicing of the precursor RNA have been observed in HIV-1 infected cells, and these can be grouped into three classes: the unspliced primary transcript (~9 kb), singly spliced RNAs (~4 kb) lacking the *gag–pol* coding region, and multiply spliced RNAs (~2 kb) lacking the *env* coding region (Purcell & Martin, 1993; Schwartz *et al.*, 1990). Approximately half of the HIV-1 RNA transcripts are unspliced and are used as message for *gag* and *pol* gene products. The unspliced RNA also serves as viral RNA, which is packaged in progeny virions. The singly spliced mRNAs encode the Env proteins and the viral regulatory proteins Vif, Vpr, Vpu and one-exon Tat, while Tat, Rev and Nef are produced from RNAs spliced at multiple sites (Purcell & Martin, 1993). The full-length RNA genome and all subgenomic messages have a common 5' methylated cap leader sequence and 3' terminal poly (A) tracts similar to eukaryotic mRNA.

Equilibrium between spliced and unspliced transcripts is critical for retrovirus replication. The splicing processes are regulated by suboptimal splice site sequences that differ to a certain extent from the optimal consensus splicing signal (Dyhr-Mikkelsen & Kjems, 1995; O'Reilly *et al.*, 1995; Staffa & Cochrane, 1994)

and splicing regulatory elements (Amendt *et al.*, 1995; Staffa & Cochrane, 1995). For example, the splicing of the first *tat* intron is down-regulated by an exon splicing silencer (ESS) located in the second *tat* exon.

Spliced as well as full length viral RNA molecules are transported to the cytoplasm and are translated into structural proteins, glycoproteins and enzymes by the host cell metabolic machinery. The multiply spliced mRNA lacks the RRE

and their transportation into the cytoplasm is Rev-independent, while unspliced and singly spliced transcripts are dependent on the viral protein Rev for transportation to the cytoplasm. In the early phase of HIV-1 gene expression, only completely (multiply) spliced mRNAs are exported to the cytoplasm, and these give rise to the Tat, Rev and Nef proteins. In the late phase of replication, Rev protein binds to the RRE sequence located in the *env* gene present on unspliced and partially spliced mRNA (Daly *et al.*, 1989; Hope, 1999) and mediates their nuclear export by delivering them into the Crm1-dependent export pathway (Fischer *et al.*, 1995; Fornerod *et al.*, 1997).

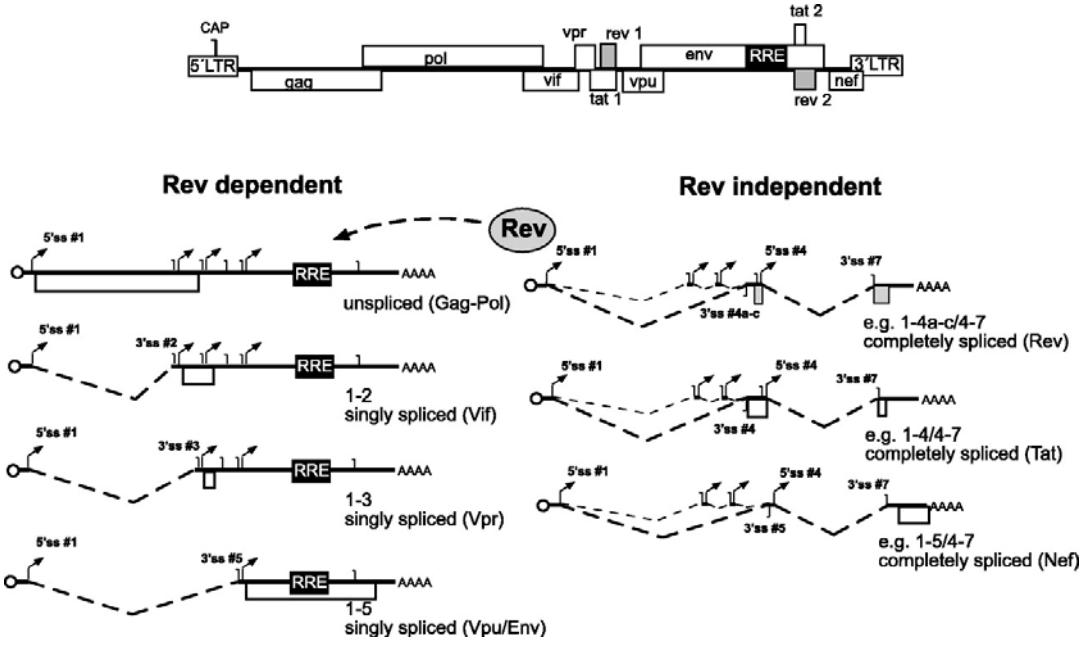

Figure 2.5. Genomic organisation of HIV-1 and splicing pattern of viral mRNAs. In the early stage of transcription, multiply spliced mRNAs encoding regulatory proteins are produced and their nuclear export is Rev independent. The unspliced and singly spliced transcripts are produced in the late stage and Rev is needed for their transport to cytoplasm. Reproduced from Bohne *et al.* (2005).

Successful infection and production of new infectious viruses requires the balanced expression of all viral genes, which is accomplished by a combination of alternative splicing, intron retention and regulated nuclear export of the primary

transcript reviewed in (Coffin *et al.*, 1997; Frankel & Young, 1998; Pollard & Malim, 1998)

Assembly and budding

Virion assembly and release from the host cell are the last stages in the retroviral replicative cycle. The structural proteins are expressed in the late phase of transcription when progeny RNA is formed also. Translation of singly-spliced *env* mRNA result in polyprotein that undergoes posttranslational modification and cleavage in the endoplasmic reticulum into an external glycoprotein (SU) and a *trans*-membrane (TM) protein inserted into the host cell cytoplasmic membrane. Gag and Pol are initially translated as a single fusion polyprotein, which is self-cleaved by the viral PR into the components of Gag (CA, MA and NC) and the components of Pol (RT, PR and IN). Gag and Gag-Pol polyprotein precursors assemble in the cytoplasm beneath the host-cell membrane and interact with the viral RNA genome forming immature virus particles close to the host cell membrane into which TM and SU are inserted.

By budding through the cell plasma membrane these particles acquire a lipid-bilayered membrane displaying Env glycoproteins. Final maturation steps in newly released extracellular virus particles involves processing of assembled Gag and Gag-Pol polyproteins by the viral protease to yield fully infectious virions.

The pathogenesis of lentivirus diseases depends on persistent viral replication in the presence of an anti-viral immune response. Two basic mechanisms are involved in this persistence: antigenic variation and restriction of viral gene expression (Cheevers & McGuire, 1988).

Genetic variation of lentiviruses

Of the three different polymerases involved in retrovirus replication, namely RT, DNA polymerase, and RNA polymerase II (Pathak & Temin, 1990), it is RT involved in the process of reverse transcription that generates the majority of the variation in the retroviral genome (Temin, 1993). The viral RT is very error-prone and lacks proof-reading ability (Preston *et al.*, 1988; Roberts *et al.*, 1988), which

results in an error frequency that has been estimated to $3x10^{-5}$ during the reverse transcription process (Mansky, 1998).

The other most important mechanism involved in the generation of variation is recombination (Hu *et al.*, 2003; Jung *et al.*, 2002; Robertson *et al.*, 1995; Temin, 1991). Recombination is believed to occur during reverse transcription as a result of the RT switching templates between co-packaged heterogeneous RNA molecules during viral DNA synthesis (Hu & Temin, 1990). It results in vast genetic alterations within the viral genome to increase the diversity of the viral population, which can improve the probability of the survival of the viral population in a changing environment (Coffin, 1979; Temin, 1991).

High virus production rate of 10^8 to 10^9 virions per day (Ho *et al.*, 1995; Wei *et al.*, 1995) and large numbers of infected cells of 10^7 to 10^8 (Offermanns *et al.*, 1997) have been reported. This continual increase in genetic diversity enables HIV-1 to rapidly adapt to a variety of selection pressures. Examples include escape from the humoral (Richman *et al.*, 2003; Wei *et al.*, 2003) and cytotoxic-T-lymphocyte (CTL) (Borrow *et al.*, 1997; Cao *et al.*, 2003; Draenert *et al.*, 2004; Price *et al.*, 1997) immune responses, as well as resistance to antiretroviral drugs (Johnson *et al.*, 2003).

In BIV the RT gene is quite conserved, with 89 % nucleotide (89 % amino acid) or greater identity, in contrast to the variable SU, with 45 % or greater nucleotide identity (51 % or greater amino acid similarity) among BIV isolates in pair-wise comparisons. The SU gene may also vary greatly in size among different viral isolates and even within viruses isolated from the same animal (Suarez & Whetstone, 1995, 1997).

The *trans*-activator of transcription, Tat

All lentiviruses encode Tat, a small RNA-binding protein which regulates the transcription of the viral genome from the LTR promoter (Tang *et al.*, 1999). Multiply spliced *tat* transcripts were produced in the early stage of HIV-1 infection

(Malim *et al.*, 1988; Purcell & Martin, 1993) even before integration (Wu & Marsh, 2003; Wu, 2004). These transcripts lead to the synthesis of a few Tat molecules sufficient to stimulate HIV transcription, leading to the production of additional *tat* transcripts and Tat protein. In HIV-1, this nuclear protein is indispensable for virus replication (Dayton *et al.*, 1986; Fisher *et al.*, 1986). Tat acts as an elongation factor of transcription through binding to TAR (Kao *et al.*, 1987); without Tat, HIV transcriptional elongation is inefficient and results in abortive transcripts that can not support viral replication (Kao *et al.*, 1987; Toohey & Jones, 1989).

There are several forms of the Tat protein. In most wild type isolates of HIV-1, Tat comprises 101 amino acids, the first 72 of amino acids encoded by *tat* exon-1 and amino acids 73–101 encoded by *tat* exon-2. Some laboratory-passaged strains, like LAI and HXB2 from HIV-1 group M, produce an 86 amino acid form. Since most wild-type strains produce the 101 amino acid form, it has been suggested that the 86 amino acid variant represents a truncated non-natural form of the protein resulting from a single nucleotide change from G to A in amino acid 87; this nucleotide change appears to generate a stop codon leading to the expression of a truncated Tat of 86 amino acids (Jeang *et al.*, 1999; Neuveut & Jeang, 1996). The preservation of residues 87–101 in wild-type isolates hints at some biological importance of this region, even if the region is not required for *ex vivo* propagation of the virus, and its importance is supported by the apparent importance of exon 2 in several biological assays (Col *et al.*, 2002; Jeang *et al.*, 1999; Neuveut & Jeang, 1996; Verhoef *et al.*, 1998).

Domains and functions of Tat

Tat protein can roughly be divided into several domains or regions, each having specific biochemical and functional characteristics (Kuppuswamy *et al.*, 1989).

Tat consists of five different domains, or dominant functional regions: the N-terminal domain, the cysteine-rich domain, the core, the basic domain and the C-terminal domain (Figure 2.6). The basic and core regions are conserved among lentiviruses (Rana & Jeang, 1999).

An acidic N-terminal domain (amino acids 1–20) forms a stable structure that contains both hydrophilic and hydrophobic amino acids arranged in a serial fashion, and in the majority of variants has five prolines which form a conserved motif $(x)_2P(x)_2P(x)_3P(x)_3P(x)_3$; the high concentration of proline in this region may be able to prevent the degradation of Tat by proteases (Jeang *et al.*, 1999; Loret *et al.*, 1991). The role of this domain in *trans*-activation has not yet been clearly defined. Introduction of mutations and deletions in this domain have given rise to contradictory results. In one study, this domain was shown to be essential and the deletion of the domain led to a loss of *trans*-activating properties (Kuppuswamy *et al.*, 1989). In another study, deletion of the domain failed to prevent *trans*-activation of the HIV genes (Sadaie *et al.*, 1988).

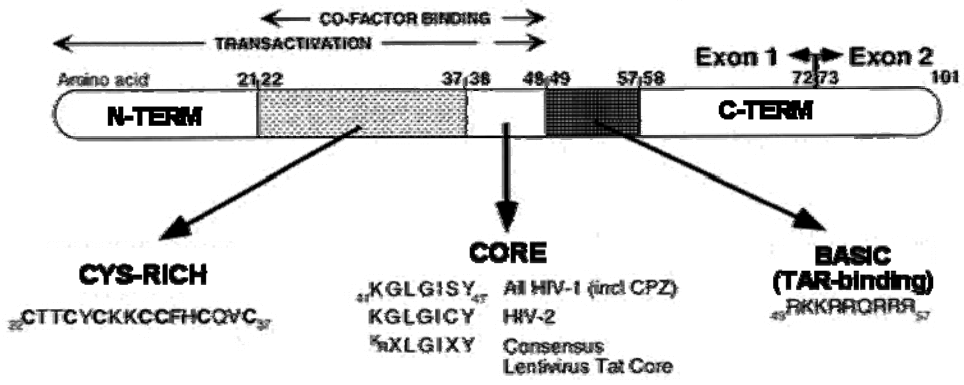

Figure 2.6. Schematic diagram of HIV-1 Tat structure. Tat protein can be divided into five physical domains; N-terminal (N-TERM), cysteine-rich (CYS-RICH), core, basic, and C-terminal (C-TERM) domains. Motifs of the functionally important domains are indicated. Adapted from Freed & Martin (2001).

The Cys-rich domain (amino acids 21–37) contains seven highly conserved cysteine residues, most of which are essential for the *trans*-activational activity of Tat and virus replication (Sadaie *et al.*, 1990). This region is responsible for the intramolecular disulfide bond formation of Tat (Koken *et al.*, 1994), and it induces HIV replication and participates in TAR-dependent *trans*-activation (Boykins *et al.*, 1999). Deletion of this domain, or induction of point mutations of certain cysteine

residues, leads to a loss in its ability to *trans*-activate HIV-1 genes (Kuppuswamy *et al.*, 1989; Rice & Carlotti, 1990; Ruben *et al.*, 1989). While it is evident that the integrity of this cysteine rich domain plays an important role in the activation of HIV-1 genes, in some studies Tat lacking both domains 1 and 2 was found to have the same level of *trans*-activating activity as the whole protein (Green & Loewenstein, 1988). It is probable that this domain is involved in a number of interactions with cellular- transcriptional co-activator that interacts with a number of DNA-binding proteins and cofactor proteins involved in the regulation of transcription (Fridell *et al.*, 1995; Hottiger & Nabel, 1998; Kamine *et al.*, 1996)

The core domain or region (amino acids 38–48) consists of a conserved and rigid α-helical structure shown to enhance the binding of Tat to TAR; it seems to be required for the specific recognition of Tat with the hairpin loop of TAR, which is formed at the start of the viral mRNA.(Bayer *et al.*, 1995; Churcher *et al.*, 1993). It has been suggested that together with the Cys-rich domain, it circumscribes the minimal activation domain of Tat (Carroll *et al.*, 1991; Derse *et al.*, 1991). Lysine 41 may be of particular importance in the *trans*-activation of HIV-1 genes, as point mutations of this have led to a dramatic diminution or loss of *trans*-activating capability (Kuppuswamy *et al.*, 1989).

The basic domain (amino acids 49–59) is also highly conserved and contains an RKKRRQRRR motif required for binding to TAR (Frankel & Clarke, 2000; Karn, 1999). It also functions as a nuclear localisation signal (NLS) by binding with importin β (Truant & Cullen, 1999). Mutations in this area lead to a loss of *trans*-activating activity (Hauber *et al.*, 1989). This is one of the domains that affixes to the hairpin loop of TAR (Aboul-ela *et al.*, 1995; Loret *et al.*, 1992; Puglisi *et al.*, 1992) and peptides that correspond to this area are capable of binding to TAR with almost the same affinity as the entire Tat protein (Calnan *et al.*, 1991; Loret *et al.*, 1992; Tao & Frankel, 1992). However, residues flanking this basic domain have been shown to significantly influence the specificity of the interaction between Tat and TAR (Karn, 1999), and this may be the case for both the glutamine rich (C terminal) and the core domain as well which are all necessary for transcriptional function of Tat. Lysine residues at positions 50 and 51 are

thought to be major substrates for acetylation (Kiernan *et al.*, 1999); a process necessary for Tat *trans*-activation of the LTR promoter. Acetylation might modulate Tat binding affinity for either TAR or other interacting transcription factors (Deng *et al.*, 2000; Kiernan *et al.*, 1999; Ott *et al.*, 1999). Prevention of acetylation by substitution of lysines 50 and 51 to alanines strongly reduced *trans*-activation (Deng *et al.*, 2000; Dorr *et al.*, 2002; Kiernan *et al.*, 1999), while a more conservative substitution with arginines exhibited a more modest effect (Pantano *et al.*, 2002). Computational analysis revealed correlation between protein structure, which was profoundly disturbed in the alanine substitutions in contrast to the highly maintained Tat conformation in lysines to arginines substitution, with the *trans*-activation activity (Mujtaba *et al.*, 2002; Pantano *et al.*, 2002).

The C terminal domain contains glutamine-rich pattern (amino acids 60–76) that forms a rigid structure that pairs with three nucleotides in the TAR loop, thus providing an additional motif in Tat able to recognise TAR RNA (Loret *et al.*, 1992). In the highly virulent subtype of HIV-1 Tat this motif forms an α-helical structure (Gregoire *et al.*, 2001) which brings the cys-rich and basic domains into close proximity during Tat-TAR binding. The rest of the domain is encoded by the second *tat* exon, the function of which is less characterised. However, mutations in this coding exon produce a virus that replicates poorly *in vivo* (Smith *et al.*, 2003).

Alignment of Tat proteins of HIV-1, HIV-2, SIVs, JDV, BIV, EIAV, CAEV, MVV, and Orf-A of FIV is shown in Figure 2.7, which allows their structural identification deduced from the pattern of conserved amino acids. It was demonstrated that Tat proteins of BIV and JDV display structural homology with their primate counterparts; they contain all 5 physical domains and share high conservation in Cys-rich and core domains. Considerable similarity was also shown by the EIAV Tat, which shares the 4 domains with a highly conserved core region, but lack of Cys-rich domain (Dorn *et al.*, 1990).

On the contrary, Tat proteins of CEAV and MVV, and Orf-A of FIV have different structural organisation; amino acid alignments denote a similar organisation of putative physical domains of an N-term acidic and hydrophobic, a central leucine-rich, and a C-term cysteine-rich (Tomonaga & Mikami, 1996; Villet *et al.*, 2003a), and they do not contain the core and basic domains. The N-term domain was proposed as the *trans*-activation domain, the central region involved in the recruitment of Fos and Jun to the Ap1 sites in the LTR and the C-term region involved in protein dimerisation and localisation to the nucleus (Villet *et al.*, 2003b) MVV and CAEV show high homology in the central and C-termi regions but the *trans*-activation domains are divergent.

Interaction of Tat with TAR

The replication cycle of lentiviruses consists of defined steps requiring several *cis* and *trans* genetic elements. Regulation of HIV gene expression involves a complex interplay between chromatin-associated proviral DNA, cellular transcription factors and the viral encoded *trans*-activator of transcription, Tat.

The viral core or basal promoter (nt –78 to –1) contains a TATAA box and three consensus SP1 binding sites. The enhancer (nt –105 to –79) carries a duplication of the 10-bp NF-kB binding sites.

The LTR promoter of HIV represents a most interesting example of molecular adaptation of a virus to the host cellular environment. The activity of the promoter is dependent on the viral *trans*-activator Tat (Berkhout *et al.*, 1989; Jeang *et al.*, 1999) and a variety of cellular transcription factors have been proposed to interact with the LTR in a cell-specific and cell activation-dependent manner (Gaynor, 1992; Pereira *et al.*, 2000). The 5' LTR is particularly important as it contains the promoter region, indispensable for the initiation of transcription.

The 5' LTR can be sub-divided into three regions: U3, R and U5. The promoter is situated in the U3 region, which contains a nucleotide sequence that is recognised by a number of cellular transcription factors: NF-κB, Sp1, the basal promoter TATA box and AP1. The R region contains regulatory elements such as

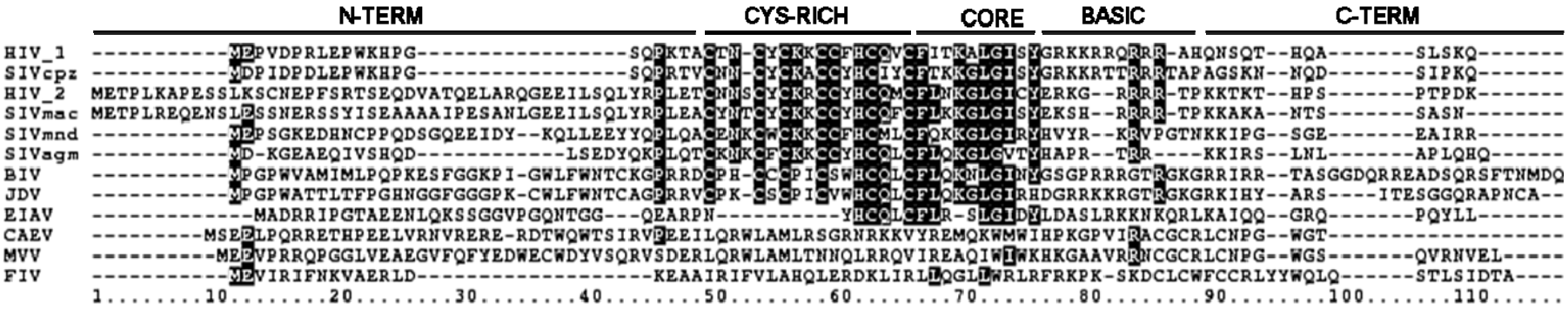

Figure 2.7. Alignment of the amino acid sequences of lentivirus Tat proteins. The deduced amino acid sequences for the Tat exon 1 are shown for HIV-1 (K03455), SIVcpz (X52154), HIV-2 (M30502), SIVmac (M19499), SIVmnd (M27470) and SIVagm (M29975). The putative N-terminal (N-TERM), cysteine-rich (CYS-RICH), core, basic, and C-terminal (C-TERM) domains of Tat are indicated. Highly conserved core domain and cysteine residues of CYS-RICH are shaded; dashes represent gaps introduced to align sequences. The GeneBank accession number of each sequence is given in brackets.

the *trans*-acting responsive element (TAR), which once translated into mRNA, folds into a specific stem-loop structure (Figure 2.8) which allows it to be recognised by Tat (Feng & Holland, 1988; Rosen *et al.*, 1985). The TAR stem contains a three-nucleotide bulge structure recognised by the arginine-rich motif (ARM) in Tat basic domain (Churcher *et al.*, 1993). This configuration is essential for *trans*-activation; mutations destabilising the TAR stem abolish Tat-stimulated transcription (Selby *et al.*, 1989). The loop region of TAR (nucleotides 30–35) is required for *in vivo trans*-activation but is not involved in binding to Tat (Dingwall *et al.*, 1990).

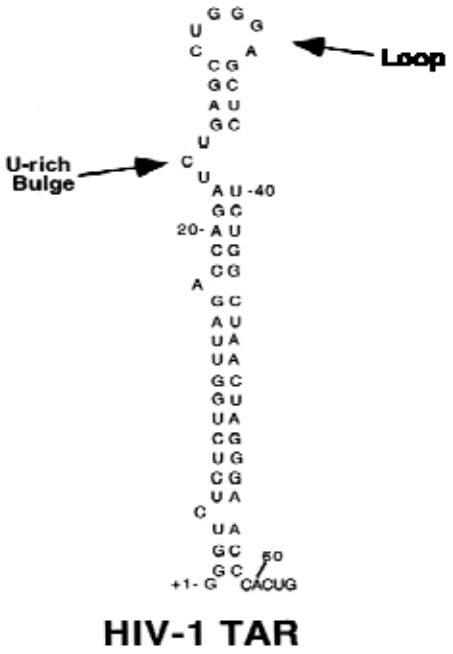

Figure 2.8. Tat responsive element stem-loop structure. The important site for Tat binding, U-rich bulge, and essential region for activation, the loop, are indicated. Adapted from Freed & Martin (2001)

Tat binds to the 5' end of the newly transcribed TAR RNA and promotes the assembly of transcriptionally active complexes at the LTR and increases the rate

of elongation by RNA polymerase II which results in the augmentation of viral gene transcription (Jones & Peterlin, 1994; Rana & Jeang, 1999). The binding requires CyclinT1 (CycT1), a cofactor that is part of the cellular-positive acting transcription elongation factor (P-TEFb), a component of pre-initiation transcription complexes which stimulates RNA polymerase II (*pol* II) elongation (Marshall & Price, 1995). Tat- CycT1 interaction is highly cooperative. CycT1 binds with both Tat via the cysteine-rich and core domains and the TAR terminal loop sequences, and remodels the structure of Tat to enhance its affinity for TAR RNA, and that TAR RNA further enhances the interaction between Tat and CycT1 (Bieniasz *et al.*, 1998; Wei *et al.*, 1998; Zhang *et al.*, 2000). CycT1 forms a heterodimer with the cyclin-dependent kinase 9 (CDK9), and recruitment of this heterodimer to the transcription initiation site leads to the hyper-phosphorylation of the carboxyl terminal domain (CTD) of the largest subunit of *pol* II, which potentially enhances the processivity of the *pol* II (Bieniasz *et al.*, 1999c; Isel & Karn, 1999; Ping & Rana, 1999). Similar mechanism was shared by SIV and EIAV Tat proteins, which demonstrate dependency on CycT1 for both TAR interaction and transcription elongation (Bieniasz *et al.*, 1999b, 1999c). The mechanism of Tat *trans*-activation is illustrated schematically in Figure 2.9.

Not all lentiviruses utilise a Tat-TAR interaction for transcription activation. In MVV, CAEV and FIV, there is no stem-loop structure within the LTR analogous to the TAR sequences. The Tat protein of MVV, and presumably that of CAEV, stimulates viral gene expression indirectly by interacting with cellular transcription factors Fos and Jun, which target the resulting complex to an AP-1 site located within the viral LTR, proximal to the transcription start site (Carruth *et al.*, 1996; Neuveut *et al.*, 1993). Once targeted to the AP-1 site, Tat recruits the TATA box binding protein (TBP) which results in enhanced transcription initiation (Morse *et al.*, 1999). This model of transcription activation is consistent with the observation that replication of MVV is enhanced in activated monocytes (macrophages) concordant with increased levels of Fos and Jun (Morse *et al.*, 1999; Shih *et al.*, 1992).

Likewise, the target of the FIV Tat-like protein encoded by the *orf*-A gene appears to be AP-1, cEBP, and ATF sites within the viral LTR (Chatterji *et al.*, 2002). Gemeniano *et al*. (2003) suggested that the *orf*-A is required for optimal viral replication and pathogenicity, but it is not critical for viral replication, as is the *tat* gene of CAEV and MVV (Villet *et al.*, 2003b); they have only mild *trans*-activating activity. The Tat proteins of CAEV and MVV are apparently incorporated into virions, localised in the nucleus and induced cell cycle arrest in the G2 phase of replication typical function of HIV-1 Vpr (Villet *et al.*, 2003a). Evidence of a similar function for the Orf-A of FIV has also been demonstrated by Gemeniano *et al.* (2004), which led to the proposal that the *tat* gene of CAEV and MVV, FIV *orf*-A code for a Vpr-like accessory protein.

Tat and reverse transcription

In addition to the *trans*-activation ability, Tat has been implicated to have an important role in the activity of reverse transcriptase. It has been observed that HIV-1 lacking a functional Tat protein (HIV-1Δ*tat*) was unable to efficiently generate proviral DNA following infection (Harrich *et al.*, 1997; Ulich *et al.*, 1999). This defect could be *trans*-complemented by co-expressing the Tat protein of not only HIV-1, but also JDV and EIAV suggesting that the reverse transcription function of Tat is conserved within lentiviruses (Harrich & Hooker, 2002). Apolloni *et al*. (2003) postulated that PR cleaved form of Tat is present in the virion and exerts a direct effect on efficient reverse transcription although the precise mechanism has yet to be investigated. On the other hand, another group argued that Tat increases overall reverse transcription activity by promoting annealing of the tRNALys onto primer binding site of viral RNA but not DNA polymerisation (Kameoka *et al.*, 2001; 2002; Liang & Wainberg, 2002). They reasoned that the interference of Tat on RT elongation might blockade premature DNA synthesis, which could be beneficial for packaging the RNA genome into the progeny virion and subsequent maturation.

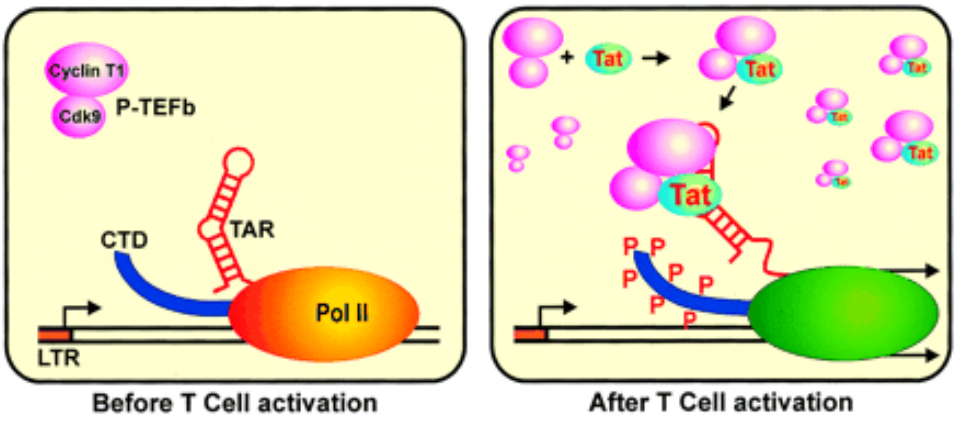

Figure 2.9. Schematic diagram illustrating Tat transactivation. Tat first recruits P-TEFb composed of CycT1-CDK9 heterodimer and binds TAR on viral transcripts. Subsequently, CDK9 stimulates the phosphorylation (P) of the C-terminal domain of RNA polymerase II (Pol II) and transcription elongation proceeds. Adapted from Price (2000).

Tat protein of BIV

BIV *tat* produces two mRNA species *in vivo*, coding for phosphorylated proteins 103 (Tat^{103}) and 108 (Tat^{108}) amino acids in length due to alternative splicing patterns (Fong *et al.*, 1997). BIV Tat contains five functionally conserved domains, a amino-terminus, cysteine-rich, conserved core, basic and carboxyl-

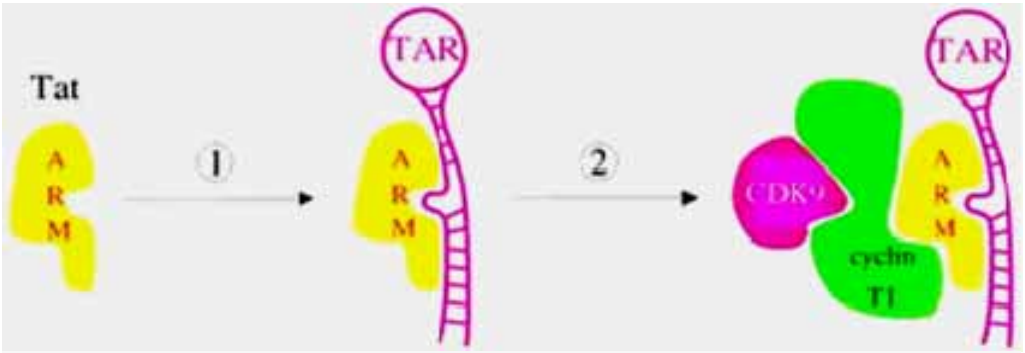

Figure 2.10. Representation of BIV Tat-TAR and P-TEFb complex. Reproduced from Barboric *et al.* (2000).

terminus domains similar to HIV (Chen & Frankel, 1994; Fong *et al.*, 1997). Functional differences have been found in the two BIV Tat species. Tat^{108} induces moderately stronger *trans*-activation of the BIV LTR than Tat^{103} (Fong *et al.*, 1997), mainly due to the presence of carboxyl-terminal regions which have been shown to increase *trans*-activation in other lentiviruses such as HIV-1 and SIV (Viglianti & Mullins, 1988).

An interesting feature of BIV Tat is that the arginine-rich RNA-binding motif (ARM) of basic domain is fully capable for binding with high affinity and specificity to the stem and bulge in its homologous TAR, and the central loop is apparently not essential for its *trans*-activating function (Chen & Frankel, 1994; Harada *et al.*, 1996; Tan *et al.*, 1993). BIV Tat-TAR has been recognised as a "primordial" lentivirus interaction, where only basic interaction and function exists (Lim & Barton, 1997; Moras & Poterszman, 1996; Srinivasan *et al.*, 1996; Ye *et al.*, 1995). This interaction does not use cellular proteins to stabilise the Tat-TAR complex and is more specific for its homologous TAR, unlike the HIV, SIV, EIAV Tat-TAR complex which necessitates Cyc-T1 (Bieniasz *et al.*, 1999b, 1999c; Gold *et al.*, 1998). Consequently, BIV Tat is able to *trans*-activate HIV-1 LTR, initiating and up-regulating viral gene expression whereas HIV-1 Tat is a poor trans-activator of BIV LTR (Bogerd *et al.*, 2000; Chen & Frankel, 1994).

Although Cyc-T1 does not appear to participate directly in BIV TAR recognition, it probably still interacts with the Tat activation domain, allowing Cdk9 recruitment, transcriptional activation, and replication (Figure 2.10) (Barboric *et al.*, 2000; Bogerd *et al.*, 2000). It has been shown that BIV Tat upregulates gene expression 15-80-fold in a TAR-dependent manner and 2.5-fold in a TAR-independent manner (Fong *et al.*, 1995; Pallansch *et al.*, 1992).

Association of Tat with pathological properties of lentiviruses

A multiplicity of functions has been ascribed to the Tat protein (Rubartelli *et al.*, 1998). Despite the lack of a signal sequence, Tat is released by infected cells and is found in detectable levels (0.01–0.1 nM) in the culture supernatants of cells

infected with HIV-1 (Chang *et al.*, 1997; Ensoli *et al.*, 1990; 1993; Westendorp *et al.*, 1994). Biologically significant amount of Tat have also been detected in the sera of HIV-1 infected individuals (Westendorp *et al.*, 1995; Xiao *et al.*, 2000). Tat is efficiently taken up by a variety of cells (Chang *et al.*, 1997; Ensoli *et al.*, 1993; Fawell *et al.*, 1994; Frankel & Pabo, 1988) and may enter T-cells by endocytosis (Vendeville *et al.*, 2004); this may lead to *trans*-activation of various cellular genes (Demirhan *et al.*, 1999b; Frankel & Pabo, 1988).

The production and release of Tat from the infected cell may activate or repress cytokines and genes that control the cell cycle in uninfected cells (Chang *et al.*, 1995). This deregulation of cellular gene expression and function by Tat cause abnormalities which may participate in AIDS pathogenesis. It is thought that Tat can affect cellular gene expression in two ways (Ensoli *et al.*, 1993; Frankel & Pabo, 1988; Tyagi *et al.*, 2001): intracellularly by direct contact with components of the transcriptional machinery; extracellularly through receptors on the surface of the cell triggering signalling pathways that will lead to a change in cell function/cellular gene expression. The extracellular Tat enters infected and uninfected bystander cells through interaction with cell membrane heparan sulfate proteoglycans (Tyagi *et al.*, 2001).

Extracellular Tat binds to chemokine receptors, integrins or CD26 to induce cellular signalling (Noonan & Albini, 2000). The binding of extracellular Tat to the T-cell activation marker CD26 has been suggested to inhibit its dipeptidyl peptidase IV activity, which is thought to mediate immunosuppressive activity (Gutheil *et al.*, 1994; Wrenger *et al.*, 1997) and induce immune cells to produce several cytokines which could lead to a dysregulation of the immune response (Nath *et al.*, 1999). In addition, Tat has been shown to induce immunosuppression by inhibiting antigen- and mitogen-induced proliferation of PBMC and T-cell clones, and by inducing immunosuppressive protein IFNα causing impairment of T-cell functions in HIV-infected individuals (Ensoli *et al.*, 1992; Viscidi *et al.*, 1989; Zagury *et al.*, 1998a). Tat has also been associated with increased IL-2 secretion in response to co-stimulation with CD3 plus CD28 in

HIV-1 infected patients which induce T-cell activation or immune hyperactivation (Ott *et al.*, 1997; Secchiero *et al.*, 2000; Westendorp *et al.*, 1994).

The expression of several cellular genes, including cytokines and their receptors, is modulated by Tat (Buonaguro *et al.*, 1992; de Paulis *et al.*, 2000; Sharma *et al.*, 1995), whereas levels of MIP1-α, an inflammatory response modulator, and IL-12 are down-regulated (Ito *et al.*, 1998; Sharma *et al.*, 1996). In microglia and astrocytes, dysregulation of chemokine and chemokine receptor expression together with the secretion of inflammatory cytokines from infected monocytes is toxic to the cells of the central nervous system and could lead to encephalitis and dementia (Conant *et al.*, 1998; McManus *et al.*, 2000).

The depletion of CD4+ T cells is the most characteristic trait of HIV infection and there can be loss of these cells in the absence of their infection, which has been ascribed to apoptosis induced by Tat (McCloskey *et al.*, 1997; Purvis *et al.*, 1995; Westendorp *et al.*, 1995). The rate of apoptosis was slightly enhanced in HIV-1 productively infected cells in comparison with uninfected or bystander cells (Azad, 2000; Bahbouhi *et al.*, 2004; Bolton *et al.*, 2002). This relatively low level of apoptosis in HIV productively infected cells has been linked to the ability of Tat with potent up-regulation of the anti-apoptotic protein Bcl2 and IL-2 (Andreas Ehret, 2001; Bahbouhi *et al.*, 2004; Bennaser & Bahroui, 2002; Buonaguro *et al.*, 1992; Ndolo *et al.*, 2002; Ott *et al.*, 1997; Scala *et al.*, 1994; Vacca A. *et al.*, 1994; Westendorp *et al.*, 1994; Zauli *et al.*, 1995a).

Tat is also responsible for the spread of infection; several postulated mechanisms have been correlated with this property. One method is its function as a chemo-attractant involving several different cell types of the immune system (Benelli *et al.*, 2000). By moving into the area of a cell productively infected with HIV, these attracted cells are more easily infected. Tat also stimulates dendritic cell (DC) maturation and induces expression of chemokines that contribute to the recruitment of activated T cells and macrophages, the targets of HIV-1 infection (Izmailova *et al.*, 2003). In addition, Tat has been implicated in up-regulating the expression of chemokine receptors such as CCR5 and CXCR4 that serve as HIV

co-receptors on lymphocytes or monocytes/macrophages, thus rendering bystander cells more susceptible to infection with M- or T-tropic viruses (Huang *et al.*, 1998; Secchiero *et al.*, 1999). And recently (Marchio *et al.*, 2005) showed that Tat segregated on the cell membrane interacts with gp120 facilitating virus attachment and entry into cells.

Tat has also been associated with the development of Kaposi's sarcoma (KS) in HIV-infected individuals. Tat promotes the growth of activated vascular and lymphatic endothelial cells, and also their migration, invasion and adhesion. It has been reported that Tat and basic fibroblast growth factor (bFGF) act synergistically in angiogenesis, inducing the growth of KS cells (Albini *et al.*, 1994; Ensoli *et al.*, 1990). Extracellular Tat induces cytokine production such as angiogenic factors in macrophages. This means that the presence of Tat leads to a production of blood vessels, which may promote the development of tumours. Extra-cellular Tat stimulates the growth of cells stemming from KS in individuals infected by HIV-1 promoting the growth and invasiveness of KS cells (Barillari & Ensoli, 2002; Cantaluppi *et al.*, 2001; Ensoli *et al.*, 1994).

Immune response to Tat

An immune response to Tat has been detected in HIV-1 infected humans and animals, as well as in animals vaccinated with Tat. Tat is not immunodominant in natural HIV infections and in HIV-infected patients the prevalence of Tat antibodies varies from less than 15 % to 35 %, and longitudinal studies have indicated that while antibody is sometimes constantly detectable it is in other patients only occasionally detectable (Krone *et al.*, 1988; Lamhamedi-Cherradi *et al.*, 1992; Lieberman *et al.*, 1997; Reiss *et al.*, 1990; Wieland *et al.*, 1990). Interestingly, a significant lower prevalence of Tat antibodies has been detected in patients with KS (Demirhan *et al.*, 1999a), and there is a low or absent antibody response to Tat in patients with p24 antigenaemia and those that have progressed to develop AIDS (Re *et al.*, 1995).

During *in vitro* infection, relatively low levels of anti-Tat antibodies clearly inhibit Tat-mediated *trans*-activation of HIV-1 LTR or intracellular trafficking of Tat (Cruikshank *et al.*, 1997; Mhashilkar *et al.*, 1995; Poznansky *et al.*, 1998), and HIV-1 replication in various cell lines and in PBMC cultures in a concentration-dependent manner (Re *et al.*, 1995; Steinaa *et al.*, 1994; Tosi *et al.*, 2000).

Induction of Tat antibody has been associated with HIV-1 induced immunosuppression of T cells, as well as HIV-1 induced generation of suppressor T cells (Lachgar *et al.*, 1996). Induction of Tat-specific antibody in vaccinated rhesus macaques was also associated with minimisation of chronic plasma viraemia (Goldstein *et al.*, 2000). Thus, extracellular Tat-antibodies may inhibit the paracrine activation pathway shown to be one feature of extracellular Tat protein (Ensoli *et al.*, 1993).

Two major domains have been associated with the immunogenicity of Tat and are most often recognised by specific antibodies (Figure 2.11) (Belliard *et al.*, 2003; Moreau *et al.*, 2004; Noonan *et al.*, 2003). Monoclonal antibodies raised against the N-terminal sequence of Tat were shown to completely inhibit Tat *trans*-activation (Demirhan *et al.*, 1999a) and to delay HIV-1 replication in PBMCs (Zauli *et al.*, 1995b). The second major immunogenic region is found in the basic domain of Tat (Goldstein, 1996) which is recognised by antibody from infected individuals (Demirhan *et al.*, 1999a; Re *et al.*, 2001a; Rodman *et al.*, 1999; Tahtinen *et al.*, 1997) and vaccinated macaque or mice (Marinaro *et al.*, 2003; Tikhonov *et al.*, 2003). Antibodies to this domain have been associated with neutralising activity *in vitro*, inhibition of extracellular Tat-dependent *trans*-activation, reduction of HIV-1 replication in acutely infected T cells, inhibition of reactivation of virus replicating at low levels in chronically infected cells (Moreau *et al.*, 2004), and neutralisation of the apoptotic effect (Belliard *et al.*, 2003).

Belliard *et al.* (2005) demonstrated that the epitopes involved displayed limited polymorphism among HIV strains, but that the neutralising activity of induced Tat antibodies failed to protect against challenge in rhesus macaques.

Another interesting function of Tat is its apparent role as a T-cell adjuvant preferentially stimulating a Th1-type response (Fanales-Belasio *et al.*, 2002a; 2002b; Ramakrishna *et al.*, 2004). Gavioli *et al.* (2004) suggests that the Tat is involved in optimising and modulating the generation of CTL epitopes that benefit Th1 response. However, contrary evidence has been presented that the role of Tat in antigen-specific immunosuppression is by a directly modulation of T-cell function (Cohen *et al.*, 1999; Viscidi *et al.*, 1989; Zagury *et al.*, 1998a).

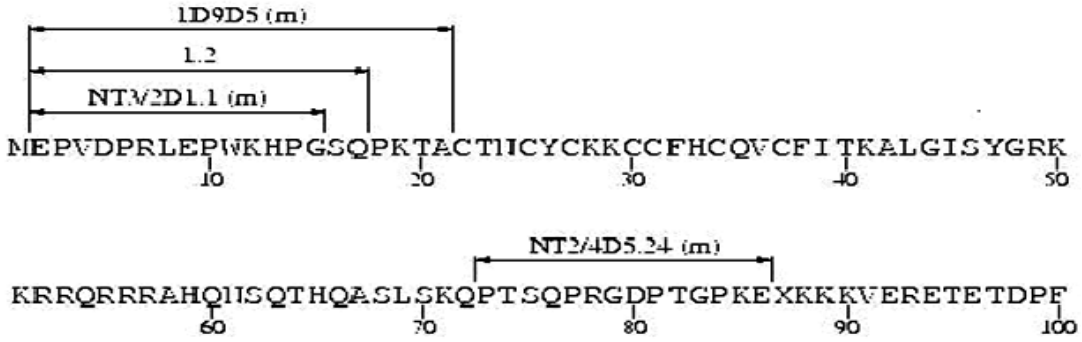

Figure 2.11. B-cell epitopes of HIV-1 Tat stimulating an antibody response. From the HIV Molecular Immunology Database (http://www.hiv.lanl.gov/content/immunology/index).

In HIV-1 infected individuals, CTL precursors (CTLp) against Tat correlate inversely with rapid disease progression to AIDS (Froebel *et al.*, 1994; van Baalen *et al.*, 1997; Venet *et al.*, 1992). Studies of a group of Gambian women who remained seronegative for HIV-1 infection despite exposure, revealed high levels of Tat-specific CTL considered to contribute to resistance of infection (Rowland-Jones *et al.*, 1995). The possibility that a CTL response against early regulatory proteins was effective in controlling lentivirus infection was also recently supported by studies on SIV infection in macaques (Pauza *et al.*, 2000; Walker & Goulder, 2000). Allen *et al.* (2000) reported that a Tat-specific CTL response induced after SIV infection in rhesus macaques was able to control primary infection, and demonstrated there was immune pressure on the Tat CTL

epitope leading to the selection of slowly replicating and apparently less pathogenic escape mutants.

Tat vaccines and vaccination

Subunit and DNA vaccines

Vaccination remains the most effective method to control viral diseases, and there have been considerable recent advances in the development of vaccines. The first generation of viral vaccines were mainly based on the use of live attenuated virus or inactivated whole virions (Plotkin *et al.*, 2004) but the recent advent of recombinant technology has accelerated vaccine development. It is now possible to use only small, well-defined immunogenic parts of pathogens, or DNA plasmids containing genes that will express proteins of interest as vaccines to induce immune responses, with the added benefit of a marked increase in vaccine safety.

The first subunit protein vaccine used was a Hepatitis B vaccine consisting of purified hepatitis B surface antigen (HBsAg) extracted from human plasma. However, identification and isolation of the gene encoding this HBsAg enabled it to be produced using recombinant DNA technology in yeast (Hilleman, 2003) replacing the plasma derived hepatitis B vaccine in human use.

The basics of recombinant DNA technology are to transfer a gene encoding an antigen, responsible for inducing immune responses sufficient for protection, to a non-pathogenic host, thereby making the production of the antigen safer and generally more efficient (Makela, 2000). Protein expression can be optimised by many methods, for example truncation of the gene may remove sequences encoding toxic peptides (Dertzbaugh, 1998), codon usage may be modified for optimal expression of the protein in the expression system such as *E. coli* (Hannig & Makrides, 1998), and the recombinant construct can be designed either for secretion or for intracellular expression of the protein. Intracellular expression in *E. coli* often leads to the formation of inclusion bodies, which may

be advantageous in that the product normally is protected from proteolytic degradation, and they are often associated with high yields of the recombinant protein. Although recombinant protein immunogens offer several advantages, they are generally poor immunogens when administered alone, and safe immune adjuvants might be needed.

The choice of expression system used will be dependent of the characteristics of the protein to be expressed, and the host used may affect the immunogenicity and protective efficacy of the product. For example, if a post-translationally modified antigen is required, a bacterial expression system is not the right choice despite the promise of a high yield (Dertzbaugh, 1998). Common major host cell systems used to produce recombinant proteins include bacteria, yeast, insect cells, and mammalian cells.

Another method of vaccination, so called DNA vaccination, has promised much but as yet no commercial DNA vaccine has been licensed for use in animals or humans. The technology is a relatively new strategy in which DNA encoding a protein of interest is incorporated into a vector and administered into a host in order to get expression of the protein by host cells with consequent induction of an immune response to the encoded antigen. Wolff *et al*. (1990) were the first to demonstrate that non-replicating DNA expression vectors could be taken up by muscle cells with *in vivo* expression of protein from the recombinant gene. Tang *et al.* (1992) demonstrated that specific antibodies were elicited following injection of plasmids encoding human growth hormone into mice. Similar studies have been repeated many times. Influenza nucleocapsid DNA vaccines primed cytotoxic T-cells (CTL) and resulted in protection from influenza challenge (Ulmer *et al.*, 1993). Other DNA vaccines have since then been reported to generate immune responses against various antigens, and also protective immunity in several diseases in animal species ranging from mice to humans (Liljeqvist & Stahl, 1999; Liu & Ulmer, 2000).

Most DNA vaccines incorporate a gene of interest into plasmid DNA of bacterial origin. The plasmids are engineered to contain a strong promoter mostly derived

from cytomegalovirus (CMV) or simian virus 40 (SV40) for optimal expression in eukaryotic cells (Gurunathan *et al.*, 2000). A polyadenylation signal (Poly-A) is also incorporated to stabilise mRNA transcripts, and translation initiation can be optimised by incorporation of a ”Kozak” translation initiation region (An *et al.*, 2000). The plasmid encodes an origin of replication which allows for growth in bacteria, and an antibiotic resistance gene for selective growth conditions. DNA vaccines thus utilise the transcriptional and translational machinery of the host cells allowing for the proper processing of the gene product that is then presented to the immune system of the host.

When administered intramuscularly, myocytes are less likely to be the primary inducers of immune responses as they lack co-stimulatory molecules (Donnelly *et al.*, 1997). DNA vaccines are thought to prime and cross-prime professional antigen presenting cells (APC), mainly dendritic cells (DC), to present the encoded antigen (Corr *et al.*, 1996; Corr *et al.*, 1999; Fu *et al.*, 1997; Ulmer *et al.*, 1996). These can be accomplished by direct transfection of APC or uptake of soluble antigen that has been secreted or released by transfected myocytes as illustrated in Figure 2.12. Priming or cross-priming of DCs will induce up-regulation of the co-stimulatory molecules that enable the DCs to process the antigen for both MHC class I and class II association for presentation to both CTLs and T helper cells (Liu, 2003).

Certain immunostimulatory motifs have been mapped in bacterial DNA to the unmethylated CpG dinucleotides flanked by two 5' purines and two 3' pyrimidines that are able to stimulate the innate immunity of the host. This sequence was shown to induce polyclonal B cell activation, maturation or activation of DCs and induction of T helper-1 (Th1) responses (Klinman *et al.*, 2004).

Immunisation with DNA vaccines offers several advantages. DNA vaccines are easy to manipulate, combination vaccines can easily be made because all components can be purified by the same procedure, endogenous antigen production can provide appropriate tertiary structure for the induction of conformationally specific antibodies and facilitate the induction of a cellular

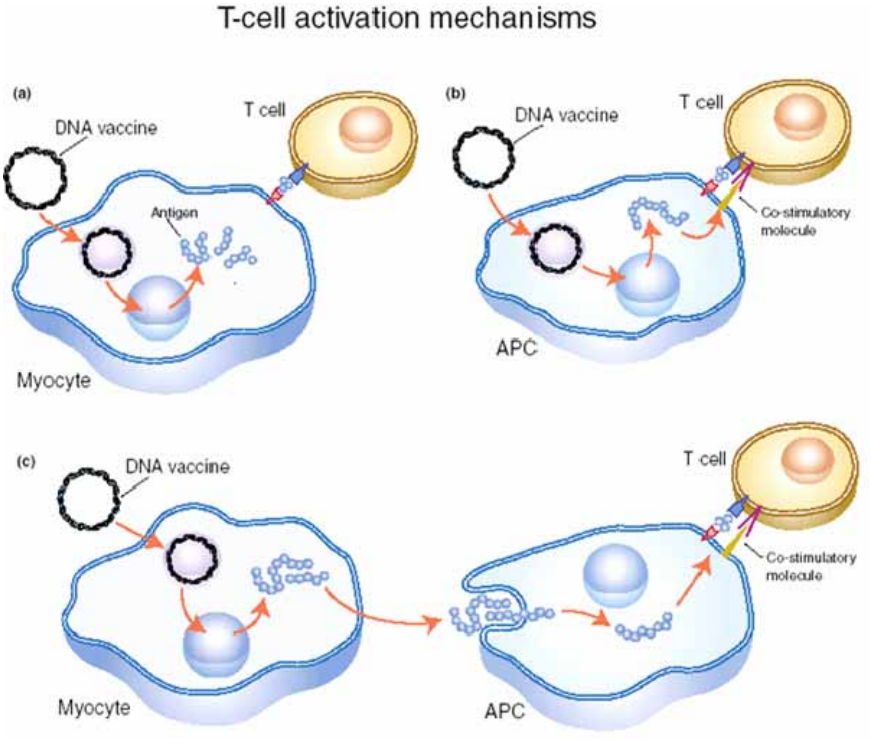

Figure 2.12. Schematic representation of the mechanism of T-cell activation by DNA vaccines. Reproduced from Liu (2003).

immune response. The same vector can also be used for subsequent immunisation since no immune response is elicited against the vector itself, in contrast to vaccinia and other live virus vectors. Moreover, sufficient quantities of plasmid DNA can be produced by simple bacterial fermentation and purification techniques, avoiding many of the complications and expense associated with the production of purified recombinant proteins. In addition, induction of immune tolerance or of autoimmunity has not generally been seen during DNA vaccination and preclinical animal studies suggest that induction of anti-DNA antibodies is unlikely (Donnelly *et al.*, 1997).

Despite the advantages, there are areas of concern with regard to DNA vaccination and it is noted that no DNA vaccine has yet been licensed for use. A major issue involved the possible integration of injected plasmid DNA into the host cell genome, and this has to be examined carefully, although it is considered

by some not to pose a significant safety concern (Ledwith *et al.*, 2000; Martin *et al.*, 1999).

Vaccination against Tat

In the extensive studies toward the control of HIV 1 infection, Tat is one of the proteins that have been considered for therapeutic intervention, including its use as an immunogen for anti-HIV-1 vaccination. There are several reasons for targeting Tat. It is produced early during the virus life cycle and is indispensable for efficient virus replication (Adams *et al.*, 1988; Arya *et al.*, 1985; Dayton *et al.*, 1986): various *tat* transcripts have been detected before viral genomic integration (Wu & Marsh, 2003; Wu, 2004). Regulatory genes including *tat* are genetically more conserved than other genes such as *env*. Tat is also antigenically conserved, and anti-Tat IgG reacts with all the functional domains of Tat (Demirhan *et al.*, 1999a; 2000). Tat is immunogenic and anti-Tat antibodies have been correlated with low plasma viral load and delayed disease progression in HIV-infected subjects (Re *et al.*, 2001b; Reiss *et al.*, 1990; Zagury *et al.*, 1998b). It is reasonable to presume that an immune response directed against this protein might prevent later steps in the virus life cycle and inhibit the release of progeny virus particles.

Candidate Tat antigens have been produced in several formats as potential vaccines, including as a peptide (Boykins *et al.*, 2000; Goldstein *et al.*, 2000), a protein (Ensoli & Cafaro, 2000), a toxoid (Goldstein *et al.*, 2000; Pauza *et al.*, 2000), a DNA vaccine (Allen *et al.*, 2002; Cafaro *et al.*, 2001; Caselli *et al.*, 1999; Hinkula *et al.*, 1997), and a recombinant virus (Allen *et al.*, 2002; Hel *et al.*, 2002).

Although the results of these studies have not been uniformly promising, some have, and the importance of anti-Tat immune responses as a correlate of protection has been established. Vaccination experiments using active or inactive Tat in animal models were shown to induce specific immune responses and confer protection against disease progression (Agwale *et al.*, 2002; Barillari *et al.*, 1999; Pauza *et al.*, 2000).

Tat-antibodies have been produced in the sera of immunised animals and these antibodies were capable of neutralising the effect of extracellular Tat on HIV-1 replication (Cafaro *et al.*, 1999; Caselli *et al.*, 1999). A significant attenuation of disease, lower viral load, plasma p24 levels, higher CD4 count, was detected in rhesus macaques immunised with chemically modified, immunogenic Tat preparation (Tat toxoid) (Pauza *et al.*, 2000). Tat toxoid was also used to immunise seronegative individuals that demonstrated a 3-fold to more than a 10-fold increase in circulating antibodies against Tat, and positive delayed type hypersensitivity skin tests and increased lymphoproliferative responses to Tat *in vitro* (Gringeri *et al.*, 1999). Low level T-cell proliferative responses to Tat has been detected in humans (Calarota *et al.*, 1999; Gringeri *et al.*, 1999), monkeys (Cafaro *et al.*, 1999; Pauza *et al.*, 2000; Putkonen *et al.*, 1998) or mice (Caselli *et al.*, 1999) immunised either with Tat protein or plasmid DNA.

The immunological responses needed for protection are still unclear. Some studies showed that both the humoral and cell-mediated responses were needed for attenuation of infection (Pauza *et al.*, 2000). Others have reported that neutralisation titres do not correlate with protection (Belliard *et al.*, 2005). A cellular immune response to the regulatory proteins of HIV-1, especially Tat, is considered important for protection against disease progression (Allen *et al.*, 2000; van Baalen *et al.*, 1997).

Various DNA vaccines have also been tested for the purpose of inducing cellular immune response. These vaccines shown to induced potent T-cell proliferative responses in monkeys (Cafaro *et al.*, 2001; Fanales-Belasio *et al.*, 2002b), mice and human (Hinkula *et al.*, 1997), and strong antibody response when combined with protein booster (Hinkula *et al.*, 1997; Putkonen *et al.*, 1998; Reiss *et al.*, 1990).

Jembrana disease

In 1964 an epidemic of an infectious disease affecting Bali cattle (*Bos javanicus*) was first reported in the Jembrana district on the island of Bali in Indonesia

(Adiwinata, 1967). The disease spread rapidly throughout Bali and within twelve months an estimated 60,000 deaths from a total population of 300,000 (20 %) occurred (Pranoto & Pudjiastono, 1967; Wilcox *et al.*, 1995).

After many years of investigation, the causative agent, which was originally believed to be rickettsia (Ressang *et al.*, 1985), has been characterised as a lentivirus, closely related to *Bovine immunodeficiency virus* (BIV), based on the antigenic cross-reactivity of CA and genomic sequence analysis (Chadwick *et al.*, 1995a; Kertayadnya *et al.*, 1993; Wilcox *et al.*, 1992), and named Jembrana disease virus (JDV). It causes an acute and severe clinical and pathological manifestation unique to Bali cattle (Chadwick *et al.*, 1995b; Dharma *et al.*, 1991; Kertayadnya *et al.*, 1993; Soesanto *et al.*, 1990; Wilcox *et al.*, 1995).

The acute nature of JD, with 100 % morbidity and 20 % fatality rates, is atypical of nearly all other lentivirus infections. After a short incubation period of 4–12 days (d), infected cattle develop consistent clinical signs of fever, anorexia, lethargy, nasal discharge, diarrhoea and lymphadenopathy that persist for about 12 d (Soesanto *et al.*, 1990). At this acute clinical stage high titres of virus of up to10^8 infectious units/mLcan be found in the plasma (Soeharsono *et al.*, 1990). The major haematological changes include leukopenia, lymphopenia, eosinopenia and a slight neutropenia, a mild thrombocytopenia, a normocytic normochromic anaemia, uraemia and hypoproteinaemia (Soesanto *et al.*, 1990).

Pathological changes reflect a rapid, intense proliferative disorder of blastic lymphocytes in the parafollicular regions of lymphoid tissues in the spleen, lymph nodes and other organs, particularly the gut. Spleen is congested and enlarged. Marked enlargement of the spleen, to 3 to 4 times normal size, and a generalised enlargement of lymph nodes was observed frequently (Budiarso & Hardjosworo, 1976; Pranoto & Pudjiastono, 1967).

The typical development of pathological changes following JDV infection can be divided into three distinguishable phases (Dharma *et al.*, 1991). Phase one changes consist of a generalised lymphoreticular reaction, which occurs during the first week post-infection (p.i.), prior to the development of clinical signs.

During the second phase, from one to five weeks p.i., the spleen and lymph nodes become markedly enlarged, petechial haemorrhages may occur on serosal surfaces, and ulcerations are sometimes found on oral and intestinal mucosae. Histopathological analysis demonstrates an intense non-follicular lymphoproliferative disorder in which pleomorphic lymphoblastoid cells come to predominate throughout parafollicular areas of the spleen and lymph nodes, causing total destruction of the normal follicular architecture. A similar proliferative cellular infiltrate is also seen in the parenchyma of the lungs, liver and kidneys and other organs, and unusual lesions containing proliferative macrophage-like cells are seen within small vessels in the lung (Budiarso & Rikihisa, 1992; Dharma *et al.*, 1991). This multi-system involvement contributes to the mortality rate of about 20 %, although earlier outbreaks often had higher mortalities (Dharma *et al.*, 1991; Teuscher *et al.*, 1981). In non-fatal infections, phase three changes represent regression of lesions and commence about five weeks p.i. These changes consist of a progressive decline in the parafollicular reaction and a marked increase in follicular development and plasma cell formation. In animals that survive the acute phase, remission of signs coincides with both a reduction in the viraemia and the appearance of anti-viral antibodies, which occurs after about 8 weeks (Hartaningsih *et al.*, 1994).

The host range of lentiviruses is normally very restricted and is limited to the natural host and closely related species. Since the initial outbreak of Jembrana disease in 1964, there has been general agreement amongst JDV investigators that the disease has attenuated somewhat, although this impression is difficult to substantiate (Wilcox *et al.*, 1995). Although some buffalo (Bubalis bubalis) were reportedly affected in an early outbreak (Pranoto & Pudjiastono, 1967), the severe effects of experimental JDV infection have been specific to Bali cattle, which are a unique species of cattle descended from wild banteng of Java (Soeharsono *et al.*, 1990; 1995). Infection of buffalo, Ongole cattle (*Bos indicus*), Friesian cattle (*Bos taurus*) and crossbred Bali cattle (*Bos javanicus* x *Bos indicus*) with the Tabanan/87 strain of JDV resulted in a transient fever and viraemia, but only a mild clinical disease.

JDV and the Tat protein

Sequencing of the complete genome confirmed that JDV is genetically closely related but distinct to BIV (Chadwick *et al.*, 1995b). The CA protein of both viruses share 75 % homology (Chadwick *et al.*, 1995b), cross react antigenically (Kertayadnya *et al.*, 1993), and no truncated recombinant proteins derived from *gag* were able to differentiate this two viruses (Desport *et al.*, 2005). With the viral genome size of 7.7 kb, JDV is a lentivirus with the smallest genome identified thus far. The genomic organisation is simpler than BIV (Figure 2.13); it contains three obligatory retroviral coding regions of *gag*, *pol*, and *env*, flanked by two long LTRs containing transcriptional regulatory elements (Chadwick *et al.*, 1995b). In addition, JDV possesses a number of accessory and regulatory genes *tat*, *rev*, *vif* and *tmx*.

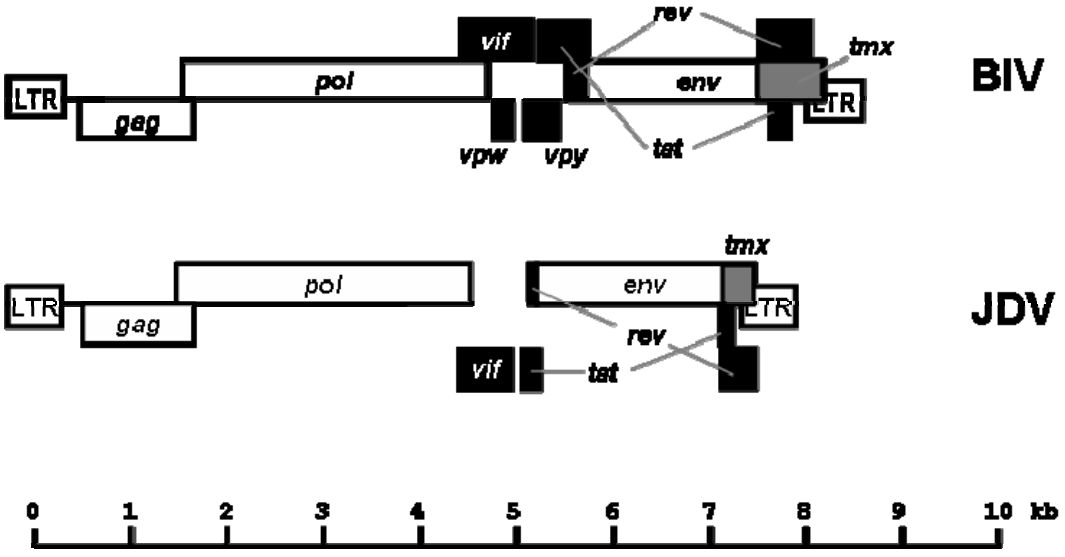

Figure 2.13. Schematic organisation of the DNA proviral genome of bovine lentiviruses.

Like the other lentiviruses, JDV encodes a Tat protein and sequence analysis has predicted that JDV Tat is translated from two separate *tat* coding exons by splicing (Chadwick *et al.*, 1995b). Alignment of the amino acid sequence encoded by *tat* exon 1 with those of BIV and the primate lentiviruses identifies the

presence of five common structural domains: acidic amino-terminal, cysteine-rich, conserved core, basic, and carboxyl-terminal (Chadwick *et al.*, 1995b; Chen *et al.*, 1999). Chen *et al.* (1999) demonstrated that the first coding exon is sufficient to generate a functional Tat, which strongly *trans*-activates the viral promoter to drive high viral gene expression that would be expected to result in the high-level viraemia seen in the disease. This activation was mediated by TAR-like elements located in the LTR, and the loop region in the TAR seemed to be less critical. The JDV Tat can not only stimulate its own LTR significantly but also stimulates BIV and HIV LTR-directed CAT expression to high levels, equivalent to those obtained with their homologous Tat proteins (Chen *et al.*, 1999). Subsequent study showed that JDV Tat *trans*-activated the HIV LTR in a partially-TAR-dependent manner (Chen *et al.*, 2000).

Similar to BIV Tat, the JDV Tat uses ARM domain to recognise its TAR sites, and adopts a ß-hairpin conformation upon TAR binding and utilise several side chains other than arginine for specific RNA recognition (Chen & Frankel, 1994, 1995; Puglisi *et al.*, 1995; Smith *et al.*, 2000; Ye *et al.*, 1995). In this situation, cyclin T1 is not required to form stable, high-affinity RNA complex and the loops do not contribute to recognition, which is in contrast to the HIV Tat-TAR interaction (Barboric *et al.*, 2000; Bogerd *et al.*, 2000; Chen *et al.*, 2000; Chen & Frankel, 1994; Smith *et al.*, 2000). Despite the structural similarity of the TAR sites (Smith *et al.*, 1998), BIV Tat binds HIV TAR only weakly and cannot use cyclin T1 as a cofactor to bind TAR. Interestingly however, the JDV Tat ARM can flexibly bind heterologous RNAs with high affinity in two distinct binding modes, by using a cyclin T1-independent, ß-hairpin conformation to bind BIV TAR and a cyclin T1-dependent, extended conformation to bind HIV TAR, utilising one arginine for specific recognition (Smith *et al.*, 2000).

The host immune responses against JDV infection

In JDV-infected cattle, an antibody response is not usually detectable until at least eight weeks p.i., which is probably a consequence of the absence of a

significant follicular B-cell response until the recovery phase (Hartaningsih *et al.*, 1994). Recovered animals do not develop any further JDV-associated clinical disease, but remain persistently infected with the virus.

The development of an antibody response to JDV was investigated in 19 cattle recovered from an experimental infection over the period of 59 weeks after infection (Hartaningsih *et al.*, 1994). An ELISA was employed to detect the antibody, using a sucrose gradient purified virus antigen prepared from the plasma of acutely infected animals. Antibody was not detected until approximately 6 weeks after infection and was present in all cattle only after 11 weeks p.i., peaked at 23-33 weeks p.i. and persisted during the observation period. The principal serological response to JDV proteins was against the p26 CA (Kertayadnya *et al.*, 1993), typical of other lentivirus infections (Battles *et al.*, 1992; Grund *et al.*, 1994; Sheppard *et al.*, 1991). An antibody response to other higher and lower molecular weight virus proteins was demonstrated by Western immunoblotting (Kertayadnya *et al.*, 1993), which could have been SU and TM glycoproteins, unprocessed Gag and other viral protein such as MA and NC as previously demonstrated in BIV infections (Whetstone *et al.*, 1990).

In another experiment, Wareing *et al.* (1999) demonstrated that JDV-infected calves gave a delayed and significantly lower antibody response when they were inoculated 3 weeks p.i. with Brucella abortus Strain 19. Similarly, compared with controls, antibody to chicken ovalbumin (cOVA) was also delayed in the JDV-infected calves.

Histopathological examination revealed atrophy of follicular (B-cell) areas and marked proliferation of lymphoblastoid cells in parafollicular (T-cell) areas, of lymph nodes and spleen (Dharma *et al.*, 1991). A scarcity of plasma cells and a significant decrease in CD4/CD8 ratio during acute phase to weeks 3 p.i. were also reported (Dharma *et al.*, 1994). After approximately 5 weeks there is a decrease in the parafollicular proliferation, regeneration of follicular cells, and increased plasma cell formation. A concurrent Pasteurella pneumonia is commonly detected in animals which die during or following the acute phase of

Jembrana disease (Dharma *et al.*, 1991). The histological changes and the delayed antibody response suggest JDV infection results in impairment of humoral immune function, which is a common aspect of the pathogenesis of disease associated with lentivirus infections, in both man and animals (Haffer *et al.*, 1990; Newman *et al.*, 1991).

The production of virus neutralising antibodies has been shown to limit virus replication and expression in many lentivirus infections (Cao *et al.*, 1995; Clements & Zink, 1996). However, there is only limited information on the presence of neutralising antibody in JDV-infected cattle (Hartaningsih *et al.*, 2001) but animals that survive the initial infection develop immunity, exhibit a minimal viraemia, and are resistant to re-infection (Soeharsono *et al.*, 1990) and there have been no reports of recurrence of disease in cattle that have survived the initial acute febrile disease. It is possible that cell mediated immune response may contribute in this resolution. This feature provides a prominent example on the success of immune response in controlling lentivirus infection.

Vaccination against JDV

Control Jembrana disease has been attempted utilising inactivated virus preparation prepared from spleen tissue of acutely affected cattle (Hartaningsih *et al.*, 2001). The vaccination procedure suppressed the duration and alleviated the severity of the disease but did not completely prevent the development of disease in animals challenged with 100 infectious doses of virus (ID_{50}). Vaccination in field conditions is restricted to endemic areas, due to concerns with the safety of the inactivated vaccine but its use has demonstrated that vaccination is feasible. There is an urgent need for a safer and more effective vaccine, and approaches using recombinant proteins and DNA vaccines should be considered using a variety of virus structural proteins and glycoproteins, and also regulatory proteins such as Tat.

Chapter 3

Jembrana disease virus tat gene: identification of transcript and sequence variation in infected Bali cattle

Summary

Transcription and sequence variation of JDV *trans*-activator gene (*tat*) was investigated in 3 strains of JDV. *tat* cDNA was cloned from spleens and PBMCs from experimentally-infected cattle during the peak period of viraemia associated with the acute disease syndrome characteristic of Jembrana disease. RT-PCR examination using a set of 7 different primers distinguished 4 predominant *tat* mRNAs expressed in similar patterns by the 3 virus strains. Nucleotide sequence analysis revealed that the transcripts were generated by multiple splicing events involving 2 potential coding exons, in the presence or the absence of noncoding exons between the major splice donor (SD) downstream from the LTR and the splice acceptor (SA) at the first *tat* exon (*tat*-1). The use of a SD downstream of *tat*-1 was common which suggests that a Tat protein of 97 amino acids specified by *tat*-1 is translated in the viraemic phase. Nucleotide sequence analysis of *tat*-1 identified limited variation; the strains from the same geographical areas shared a 96.6 and 100 % homology, but there was a lower of 89.1 % between strains from geographically different areas of Indonesia.

Introduction

Genetic organisation of lentiviruses and including the bovine lentiviruses *Jembrana disease virus* (JDV) and *Bovine immunodeficiency virus* (BIV) is characterised by the presence of retroviral obligatory genes *gag*, *pol* and *env*, along with regulatory and accessory genes (Coffin *et al.*, 1997). Lentiviruses encode transcriptional *trans*-activator (Tat) proteins that potently upregulate the level of gene expression from their cognate long terminal repeat (LTR) promoters (Cullen, 1991; Jones & Peterlin, 1994; Jones, 1997). They use a complex splicing strategy to generate different species of viral mRNA for protein production. The Tat protein is derived from multiply spliced mRNAs involving 2 coding exons; composition of the encoded protein depends on the splicing patterns between the coding exons (Fong *et al.*, 1997; Unger *et al.*, 1991). In JDV, 2 separate *tat* exons have been identified (Chadwick *et al.*, 1995b). The first exon encoded a cysteine-rich region, a core region, and a downstream basic domain that was found in the Tat proteins of most lentiviruses, including BIV (Garvey *et al.*, 1990).

JDV infection causes an acute disease syndrome in Bali cattle with a case fatality rate of approximately 20 %. Jembrana disease is endemic in Indonesia. Vaccination using a detergent-inactivated virus preparation prepared from tissues of acutely affected cattle has been shown to induce a protective immune response (Hartaningsih *et al*., 2001). Use of this vaccine is limited to areas where the disease is endemic because of uncertainty about complete inactivation of the virus (Dr Nining Hartaningsih, Personal Communication) and a safer vaccine is needed.

Attempts to control lentivirus infections in other animal systems have involved a number of approaches, including Tat vaccines. Vaccination with HIV-1 Tat in animal models has been shown to induce a protective immunity against disease progression (Agwale *et al.*, 2002; Cafaro *et al.*, 1999; Pauza *et al.*, 2000; Stittelaar *et al.*, 2002). Tat is also a potential immunogen for the control of

Jembrana disease and there is no information available on the genetic variability of *tat* that might lead to variation in antigenicity of the encoded protein.

Preliminary to an investigation of Tat as a potential immunogen for the control of Jembrana disease, further characterisation of Tat was considered necessary and is reported in this Chapter. The investigations reported include the mechanism of *tat* transcription in acutely infected animals, whether both or only one of the 2 *tat* exons was involved in the translation of the encoded Tat protein. Splice donor and SA sites were also examined in subgenomic *tat* mRNA. The variability of *tat* was investigated by an examination of proviral DNA extracted from tissues of affected animals in different regions of Indonesia and in different time periods.

Materials and methods

Isolation of nucleic acid

Extraction of total RNA from tissues

Total RNA was extracted from spleen tissues and PBMCs using a Totally RNA^{TM} kit (Ambion), which is based on a method described by Chomczynski and Sacchi (1987), according to the manufacturer's instructions. Briefly, cells or tissues were homogenised with 10 volumes of Denaturation Solution, lysates were transferred into a fresh tube (referred to as the Starting Volume). The lysate was pipetted up and down to help solubilise the RNA, and then an equal volume of Phenol:Chloroform:IAA was added. The mixture was vigorously shaken for 1 min and then kept on ice for 5-10 min and subsequently centrifuged (12,000 *g*, 10 min, 4°C). The resulting aqueous phase was transferred to a fresh tube and mixed with 1/10 volume of 5 M sodium acetate. One Starting Volume of Acid-Phenol:Chloroform was added followed by vigorous shaking for 1 min. Preparations were kept on ice and centrifuged as before. The aqueous phase was placed in a fresh tube, mixed with an equal volume of isopropanol and kept at –20°C for 1 h. After centrifugation (12,000 *g*, 15-20 min), the RNA pellet was washed with 70 % ethanol and resuspended in 0.5-1 mLof RNase-free dH_2O. To

remove residual genomic DNA, a 1/10 volume of DNase Reaction Buffer and 1 µl of RNase-free DNase I per 100 µl RNA were added, and mixed gently. Following incubation for 30 min at 37°C, a 1/10 volume DNase Inactivation Reagent was added, mixed, and incubated for 2 min at room temperature. The mixture was centrifuged (10,000 *g*, 1 min) and the supernatant transferred into a new tube and stored at -70°C until needed.

Preparation of genomic DNA

Genomic DNA was prepared by a technique similar to that for RNA except that Acid-Phenol:Chloroform was replaced with Tris-buffered Phenol:Chloroform and the resulting DNA pellet was dissolved in TE buffer (10 mM Tris, 0.1 mM EDTA pH 8).

Plasmid purification

Qiagen Miniprep kits were used for purifying plasmid DNA for subsequent sequence analysis. Briefly, 1.5 mLof an overnight bacterial culture was centrifuged (14,000 *g*, 20 seconds) and the pellet resuspended in 250 µl of buffer P1. The cells were lysed with 250 µl buffer P2, neutralised with 250 µl buffer P3 followed by gentle inversion until a precipitate formed. The mixture was centrifuged (14,000 *g*, 10 min) to remove cell debris, and the supernatant containing the plasmid DNA was applied to a QIAprep column (Qiagen). After centrifugation (14,000 *g*, 30 seconds), the flow through was discarded and the column was washed twice with 500 µl buffer PE by centrifugation (14,000 *g* for 30 s and then 2 min). Finally 60 µl of warm distilled H_2O was added, centrifuged (14,000 *g*, 1 min) and the supernate containing DNA stored at –20°C. Aliquots were subjected to agarose gel to check integrity and were also quantified fluorometrically.

Recovery of DNA from agarose gels

Following agarose gel electrophoresis, DNA fragments (bands) of interest were recovered from 0.8 % gels using QIAquick Gel Extraction Kit (Qiagen) according to the protocol of the manufacturer. Each band was excised from the gel using a

clean sharp scalpel and transferred to an eppendorf tube. Two times the gel volume of buffer QE was added and incubated at 50°C for 5 min to dissolve the gel. After complete solubilisation, the mixture was passed through the spin column by centrifugation and washed once with 750 µl buffer PE. The DNA was eluted from the membrane by adding 30 µl of warm 10 mM Tris-HCl, pH 7.5, and then centrifuged (14,000 *g*, 1 min).

Agarose gel electrophoresis

Agarose gel electrophoresis was used to fractionate DNA molecules to enable analysis of PCR products and restriction enzyme digests, to determine plasmid integrity, and estimate DNA concentration. Prior to loading, DNA samples were mixed with 6X tracking dye (0.25 % bromophenol blue, 0.25 % xylene cyanol FF and 10 % glycerol) and electrophoresed on 0.8 to 2 % agarose gels containing 0.1 µg/mLethidium bromide. Size markers (100 bp or 1 kb plus DNA ladders; Gibco) were added to separate lanes. Electrophoresis was carried out in TAE buffer (40 mM Tris, 20 mM sodium acetate, 2 mM Na_2EDTA, and glacial acetic acid to pH 8.3) at a constant voltage of 40–100 kV for 40 min to 3 h depending on the resolution required. Sometimes, variations in the gel percentage, voltage and run times were used to obtain better separation of products, especially when multiple products were expected. Gels were examined with an ultraviolet transilluminator to visualise DNA bands.

Estimation of nucleic acid concentration

The amount of RNA in samples was estimated photometrically by measuring absorption of the samples at 260 nm. An OD_{260} of 1 was considered to indicate 40 µg/mLof single-stranded RNA. The purity was determined by the OD_{260}/ OD_{280} ratio: ratios of less than 2.0 were considered to indicate the present of contaminants.

The concentration of DNA was determined by fluorometric analysis in a DYNAQuant 200 Fluorometer (Hoefer-Pharmacia). DNA samples were mixed

with 2 mLfluorometer working solution (0.1 mg/mLfluorochrome [Hoechst 33258] in 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4. The dye binds to DNA and fluoresces at 460 nm relative to the DNA concentration standardised with a known concentration of calf thymus DNA (Promega).

Oligonucleotide primers

The oligonucleotide primers in this study were designed based on the genomic RNA sequence of the Tabanan/87 reference strain of JDV (Chadwick *et al.*, 1995b). Primers were obtained from Invitrogen and dissolved in water to a final concentration of 200 µM. The sequence and annealing positions of the primers used are indicated in Table 3.1. The location of primers used for cDNA synthesis and detection is depicted in Figure 3.5.

Reverse transcription

cDNA was synthesised with Moloney murine leukaemia virus reverse transcriptase (MLV RT) SuperscriptII RNAseH^- (Invitrogen) with an antisense primer j7730/3' (Table 3.2) following the protocol recommended by the manufacturer. Briefly, total RNA (4µg) was mixed with 4 pmol primer and 10 µmol DNTP mix in a total volume of 10 µl. To disrupt the secondary structure of the RNA, the mixture was heated at 65°C for 10 min, then quickly chilled on ice for 1 min. After brief centrifugation, the following components were added: 2 µl 10X RT buffer, 2 µl 0.1 M DTT, 4 µl 25 mM $MgCl_2$ and 1 µl RNaseOUT (40 U/ µl). The content of the tube was mixed gently and incubated at 42°C for 2 min. One µl of reverse transcriptase (50 units) was added, and further incubated for 40 min. The reaction was terminated by heating at 70°C for 15 min, then chilled on ice for 2 min, and finally treated with 1 µl RNase H at 37°C for 20 min to degrade the RNA template. The reaction was stored at –70°C until needed.

Polymerase chain reaction (PCR)

PCR was employed to detect *tat* sequences in cDNA and proviral DNA, and for detection of recombinant plasmids.

Table 3.1. Oligonucleotide primers used for the detection of tat transcripts (Primers 1-7) and proviral DNA (Primers 8 -10).

Primer	Sequence (5' to 3')	Genomic positions[1]	
		Nucleotide	Region
j129/5'	TGTCTCCTCGACCCGAAC	129–146	U5-LTR
j5047/5'	GGCACAATGGAGGATTT	5047–5063	5' *tat* 1
j7069/3'	TTTGAGCGCCCAAGTTTG	7069–7052	middle *tat* 2
j7730/3'	CCGAAAGCCAAACGACCTT	7730–7712	R-LTR
Tab_Pulj3'[2=]	CACTGGAGGAC^CGTGATC	7043–7033^5335–5329	3' *tat* splice junction
Kalj1/3'[2=]	ACTGGAGGAC^CGTTATCCT	7042–7033^5335–5327	3' *tat* splice junction
Kalj2/3'[2=]	TGAGGATAAG^TCTGGAGTAGAT	6967–6958^5278–5267	3' *tat* splice junction
j4938	GGGAAGGAGCCAACGATTC	4938–4956	upstream *tat* 1
j5338	CACCGTGATCTTCCAGGGT	5338–5320	downstream *tat* 1
jK5337[3=]	ACCGTTATCCTCCAGGACCCA	5337–5317	downstream *tat* 1

[1] The position was based on JDV Tabanan/87 genomic RNA sequences (Chadwick *et al.*, 1995b).

[2] Primers were designed following sequence identification of splicing junction between the two *tat* exons. Sequences and numbers are separated by a carat (^) to indicate fusion of two separate regions of the viral genome.

[3] Primer was designed based on the Kalimantan sequences identified from the transcripts

PCR using proof-reading polymerase

Expand ™ High Fidelity PCR System (Roche) a blend of *Taq* and Pfu proof reading polymerases was used for cloning. PCR reactions were performed in a 20 µl volume containing 200–600 µM dNTPs, 5–20 pmol primers depending on the template (Table 3.2), 2.0 µl of PCR buffer with 15 mM $MgCl_2$, 2 U Expand ™ and 1–2 µl template. Amplification was performed in a PE2400 automatic thermocycler (Perkin-Elmer Cetus) under the conditions described in Table 3.3.

Table 3.2. Various PCR reaction mixes used for the detection of *tat*.

Ingredient	Volume (µl)		
	Expand™ system		Dynazyme system
	cDNA	Proviral DNA	
10X buffer with $MgCl_2$	2	2	2
dNTP mix (2 mM)	6	2	2
Forward primer (20 µM)	1	0.25	0.25
Reverse primer (20µM)	1	0.25	0.25
Polymerase	0.75	0.75	0.25
Template	2	1–2	2
Ultrapure H_2O	Up to 20 µl	Up to 20 µl	Up to 20 µl

PCR for screening of recombinant plasmids

PCR was conducted with a Dynazyme (Finnzyme) system summarised in Table 3.2 using 5 pmol of each vector-specific M13/F (5'GTAAAACGACGGCCAGT 3') and M13/R (5'AACAGCTATGACCATG 3') or gene specific primers (Table 3.1), and subjected to similar condition to the Expand ™ system but with modified annealing, at 56°C for M13 or 60°C for gene-specific primers, and elongation for 40 sec (Table 3.3).

Table 3.3. Various thermocycling conditions used for the detection of *tat*.

Step	Temp (°C)	Duration			Cycles
		Expand™ system		Dynazyme system	
		cDNA	Proviral DNA		
Initial denaturation	95	5 min	5 min	5 min	1
Denaturation	95	40 sec	40 sec	40 sec	30
Annealing	60	30 sec	30 sec		
	56 or 60[1]			30 sec	
Elongation	72	1.5–3 min	1.5 min	40 sec	
Final elongation	72	7 min	7 min	7 min	1

[1] Varied depending on the primers used in the reaction.

Cloning of PCR products

Taq polymerase has a terminal transferase activity that results in the non-templated addition of a single nucleotide to the 3'-ends of PCR products. In the presence of all 4 dNTPs, dA is added preferentially. This terminal transferase activity is the basis of the TA-cloning strategy, which in the current study was conducted using pDrive (Qiagen) or pCR2.1 (Invitrogen) cloning vectors according to the protocol provided by the manufacturers. Reaction mixtures (Table 3.4) of 10 µl containing 30–50 ng of DNA, 50 ng vector, ligase and dH_2O were mixed gently and incubated at 14°C for 2 h (pDrive) or 14 h (pCR2.1).

Table 3.4. Quantities of reagents incorporated into TA-cloning reactions.

Ingredient	Volume	
	pCR2.1	pDrive
Vector (50 ng/µl)	1 µl	1 µl
PCR product	30–50 ng	30–50 ng
Buffer	1 µl	5 µl
T4 DNA Ligase	1 µl	-
Ultrapure dH2O	Up to 10 µl	Up to 10 µl

Transformation of plasmids into *Escherichia coli*

The *Escherichia coli* Top10F' [F´{*lac*I^{q} Tn*10*(Tet^{R})} *mcr*A D(*mrr-hsd*RMS-*mcr*BC) f80*lac*ZDM15 D*lac*X74 deoR *rec*A1 araD139 D(ara-leu)7697 *gal*U *gal*K *rps*L (Str^{R}) *end*A1 *nup*G] (Invitrogen) was used in these experiments. Transformation was conducted by gently mixing 25 µl TOP 10F' one shot competent cells (Invitrogen) with 2 µl ligation mixture, followed by incubation on ice for 30 min. The cells were then heated at 42°C for 30 sec and kept on ice for a further 2 min before adding 125 µl of SOC medium. The cells were incubated at 37°C for 1 h to allow antibiotic resistance expression and then spread on Luria-Bertani (LB) plates (1 % Bacto-tryptone, 0.5 % yeast extract, 1 % NaCl and 1 % agar) containing 100 µg/mLampicillin, 50 µM isopropyl β-D-thiogalactopyranoside (IPTG) and 0.4 % X-Gal for "blue-white" screening. The plates were incubated at 37°C for at least 18 h and then kept 2 h at 4°C before further screening.

Screening for recombinant plasmids

For each cloned PCR product, 25 white colonies were randomly picked and each was diluted with 20 µl of 0.1 % Triton-X in TE buffer, and boiled for 5 min. After centrifugation at 14,000 *g* for 5 min, 2 ul of supernatant was used as a template for PCR with Dynazyme (Finnzyme) as detailed above.

Sequencing

Nucleotide sequencing was conducted by a dideoxy-chain termination method as described by Sanger *et al.* (Sanger *et al.*, 1977) in an ABI377A fluorescence automated sequencing apparatus. Half sequencing reactions were prepared for both anti-sense and sense strands using universal M13 primers in ABI PRISM^{TM} Big Dye Terminator version 3.1 ready reaction kit with Amplitaq DNA polymerase (Perkin-Elmer, Applied Biosystems). In brief, 10 µl reactions containing 500 ng plasmid DNA, 4 µl Terminator Ready Reaction mix and 3.2 pmol of M13/F or R primer were subjected to amplification conditions of 96°C for 2 min; followed by 30 cycles of 96°C/10 sec, 55°C/5 sec and 65°C/4 min, with a 14°C final hold. DNA was precipitated by adding 25 µl of 95 % ethanol, 1 µl of 125 mM EDTA and

1 µl of 3 M Na-acetate (pH 4.6) on ice for 20 min before centrifugation at 14,000 *g* for 30 min at room temperature. Pellets were washed with 70 % ethanol, vacuum-dried briefly, and submitted for sequencing. SeqEd™ version 1.0.3 (Applied Biosystem Inc.) was used to display and edit raw sequence data.

Isolation and identification of *tat* transcripts

Virus strains used for characterisation of tat transcripts

Three JDV strains were used in this study. All originated from Indonesia, two from Bali and one from Kalimantan. The 2 Bali strains included the prototype strain, Tabanan/87 isolated during 1987 from an affected animal in the Tabanan district, for which complete sequence data was available (Chadwick *et al.*, 1995b). The second Bali strain (Pulukan/00) was from an affected animal in the Pulukan subdistrict in 2000. The Kal/00 strain was obtained from an affected animal during an outbreak in the Tanah Laut district in mid-2000. The JDV strains were maintained by passage of spleen tissue in the natural host as described by Soeharsono *et al*. (1990). Information on the number of passages of each strain prior to the collection of tissues used in these current experiments was not available but was estimated at less than five.

Animal infection and processing of tissues

To enable the collection of tissues from infected animals, the 3 strains were each inoculated into a single Bali animal of approximately 18 months age at the Disease Investigation Centre in Denpasar, using procedures similar to those described by Soeharsono *et al.* (1990). Rectal temperature, total leukocyte counts and enlargement of the superficial lymph nodes were monitored. A rectal temperature in excess of 39.5°C for 2 or more days, in conjunction with leukopenia and enlargement of the superficial lymph nodes was used as an index of the occurrence of Jembrana disease. To collect peripheral blood mononuclear cells (PBMCs), heparinised whole blood was obtained and the cells separated using Ficol-paque (Gibco-BRL), then washed in phosphate-buffered saline (PBS) and pelleted by centrifugation (3,000 *g*, 5 min). The animals were killed on the

second day of the febrile period following infection, and spleen tissue was collected. Spleen tissue was examined histologically following necropsies to verify the animals were affected with Jembrana disease (Dharma *et al.*, 1991). Aliquots of the spleen tissue were washed with PBS and stored in liquid nitrogen pending further examination. In addition to the samples from experimentally infected cattle, one additional sample (Kal/03) of spleen tissue was obtained from an infected Bali animal during an outbreak in the Tanah Laut district of Kalimantan in 2003.

RT PCR

Total cellular RNA was extracted from 1 *g* of spleen or 50 µl of PBMC suspension using a Totally RNA^{TM} kit as detailed previously. Four microgram of RNA were reverse transcribed at 47°C for 40 min using primer j7730/3' which corresponds to the transcription termination region. For the detection of *tat* transcripts, 2 µl of RT product was used as a template for PCR containing 200 µM dNTPs, 20 pmol primers, 2.0 µl of PCR buffer with 15 mM $MgCl_2$ (Roche), and 0.75 µl of Expand^{TM} system (Roche). Primer pair j5047/5' and j7069/3' located in *tat* exons 1 and 2, respectively, were selected to determine the junction region. To characterise the 5' region of the transcripts following the identification of the junction sequences, junction primers Tab_Pulj3' specific for Tabanan/87 and Pulukan/00 strains, and Kalj1/3' and Kalj2/3' for Kal/00 strain, were designed and used in combination with j129/5' situated downstream the transcription start site.

Amplified PCR products were extracted after agarose gel electrophoresis and TA-cloned as described above. The cloned DNA was subjected to automated sequencing using universal M13 primers in both orientations.

Cloning of *tat* DNA and nucleotide sequence analysis

Total DNA was prepared from PBMCs and/or spleen tissues of experimentally and naturally infected cattle as described previously. The *tat* exon 1 was amplified from 300 ng of total DNA using oligonucleotide primers (Table 3.1) j4938/j5338 for Tabanan/87 and Pulukan/00, or j4938/jK5337 for Kal/00 DNA

templates in conjunction with the Expand™ system. The reaction mixtures were prepared as detailed in Table 3.2 and subjected to 30 cycles of melting at 94°C for 30 seconds, annealing at 60°C with a 1.5 min extension at 72°C. PCR products were separated on 0.8 % agarose gels and purified with a QIAquick gel extraction kit (Quiagen). The resulting DNA fragments were TA cloned and sequenced using universal M13 primers as described earlier. Sequences were aligned with PileUp program of the Genetics Computer Group (GCG) (Wisconsin package) and displayed by Pretty program provided by ANGIS (Australian Genomic Information Services).

Table 3.5. Rectal temperatures at intervals after cattle were infected with 3 JDV strains. A rectal temperature in excess of 39.5°C was indicative of the onset of the acute phase of Jembrana disease. The animals were killed on the second day of the febrile reaction. Samples collected from the animals for subsequent analysis are indicated.

Days after infection	Temperature (°C)			Samples collected for analysis		
	Tab	Pul	Kal	Tab	Pul	Kal
1	38.0	38.6	38.2	–	–	–
2	–	38.8	–	–	–	–
3	38.3	38.9	38.4	–-	PBMCs	–
4	38.0	38.7	38.3	PBMCs	–	PBMCs
5	38.0	38.5	38.2	–	PBMCs	–
6	38.3	39.9	38.6	–	–	–
7	39.2	†	38.3	PBMCs	Spleen, PBMCs	PBMCs
8	39.9		39.3	–	–	–
9	†		39.9	Spleen, PBMCs	–	–
10			†	–	–	Spleen, PBMCs

† denotes the animal was killed.

Results

Isolation and identification cDNAs representing 3' end of *tat* transcripts

Genomic sequence analysis has predicted that JDV *tat* is encoded by 2 exons (Figure 3.5A). The first is located downstream of the *vif* and overlapped at half 3' end with *env* from nt 5010 to 5303, and the second is encoded by an exon located within the envelope gene from nt 7057 to 7087. In order to identify splicing patterns between the 2 coding exons, 3 different primers were used to amplify spleen and PBMC mRNAs from experimentally infected cattle. Primer j5047/5' was complementary to 5' to the first *tat* exon, and the second primer, j7069/3' was homologous to the predicted second exon. Primer, j7730/3' was used in place of j7069/3' to hybridise at the poly(A) tail located at the LTR. Clones were generated either with the first (j5047/5'and j7069/3') or the second (j5047/5'and j7730/3') pair of primers. Products greater than 300 bp were amplified with the first primer pair (Figure 3.1A), while the second primer set allowed amplification of 3' end transcripts of approximately 1,000 bp size (Figure 3.1B). Productive amplification was detected only from spleen and PBMCs collected on the second day of fever. No amplicon was obtained from PBMCs collected before fever. To resolve nt sequence of this region, the PCR products were TA-cloned; 25 clones were randomly selected from each amplification reaction and screened by PCR using j5047/5'– j7069/3' primer pair. In 300 white colonies, 2 different PCR products were detected (Figure 3.1, A and B), the predominant product from both spleen and PBMC cDNA of the 3 JDV strains were clones that produced band of approximately 300 bp as shown in Figure 3.2. One band migrating slightly above the major band was detected from Kal/00 sample (Figure 3.2C lane 6). Subsequent nucleotide sequencing confirmed the size of the bands, which were 327 bp for the predominant band and 345 bp for the other. Both incorporated coding exons 1 and 2 but displayed different splice sites between these 2 exons. The majority of transcripts demonstrated the splice junction generated by joining the SD downstream of coding exon 1 at nt position 5335 and the SA at the start of exon 2 at nt position 7033; these were conserved

among the 3 strains despite nt substitutions detected in the Kal/00 strain (Figure 3.7). The only transcript detected from the Kal/00 strain utilised a SD upstream the stop codon of exon 1 at nt 5268 and a SA upstream of the second exon at nt 6947. Amplification with the second primer set in all clones also demonstrated the extension of the second coding exon to the 3' end of the JDV genome, right through the polyadenylation site at nt 7730.

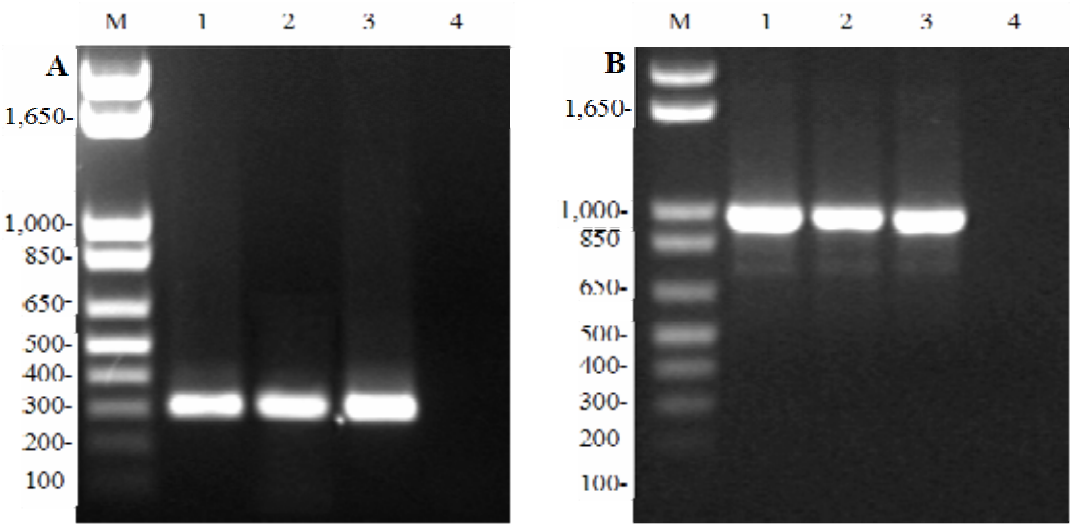

Figure 3.1. PCR amplification of *tat* cDNA. To identify 3' regions of mRNA and the splice patterns between the 2 coding exons, cDNA was amplified using primer sets (A) j5047/5' and j7069/3' and (B) j5047/5' and j7730/3', which detected products of approximately 300 bp and 1000 bp, respectively. Each PCR product was subsequently TA-cloned and sequenced. Lanes 1-3, Tabanan/87, Pulukan/00 and Kal/00 strains, respectively. Lane 4, control without DNA. Lane M, 1 Kb Plus DNA ladder (Invitrogen).

Isolation and identification of 5' end of transcripts

Following identification of splicing sites between the 2 *tat* exons, three 3' "junction" primers were designed. These were Tab_Pulj3', Kalj1/3' and Kalj2/3', the sequences of which spanned the splice junctions created by joining the SD at nt position 5335 and SA at position 7033 (5335^7033) with Tabanan/87 and Pulukan/00 strains, and 5335 to 7033 (5335^7033) and 5278 to 6967

(5278^6967) with the Kal/00 strain. A 5' primer (j129) homologous to the U5 region of the LTR and each of junction primers mentioned above was used to synthesise single-stranded cDNA from RNA extracted from tissues of the JDV-infected cattle. In combination with primers complementary to the common splice sites 5335^7033, 6 products varying from 490 to 706 bp were amplified, whose separation was achieved through electrophoresis in 2 % agarose gels at 40 V for 4–5 h as shown in Figure 3.3A. In contrast, combination of j129 and a primer homologous to the second junction sequence (5278^6967) of Kal/00 amplified a different region of the genome. Sequence analysis of this product (Figure 3.3, Panel B) showed there was considerable homology between the junction primer and 5' region of the genome (Figure 3.3, Panel C). Attempts to increase the specificity of the reaction by using higher annealing temperature did not result in amplification of the expected product.

PCR products generated with the first primer set were TA-cloned and screened by PCR with the same primers used for cDNA amplification. In 300 randomly selected clones, 6 types of PCR product were detected (Figure 3.4). The predominant types were 583 (type III), 553 (type IV), 515 (type V) and 490 (type VI) bp as shown in Figure 3.3, Panel A). Larger 706 (type I) and 638 bp (type II) products (Figure 3.3, Panel A) were detected but at a considerably lower frequency than the smaller transcripts and they were detected only in the tissues of animals infected with the Pulukan/00 strain (Fig 3.4, Panel B, lanes 1–3 and 17). The various transcripts differed in SA sites used (nt 4938 in types I, II IV and VI; nt 4894 with types III and V) upstream of the first coding exon, and in the presence (nt 4014–4081 in types I, III and IV; nt 4663–4808 in types I and II) or absence (types V and VI) of non coding exons between the first major splice site at nt 204 and the first *tat* exon, as illustrated in Figure 3.5, Panel B. Evidence of splicing between the LTR region and *tat*-1 of a truncated *tat* transcript (type VII) detected in Kal/00 was not obtained. The SD and SA sites utilised to generate the different *tat* transcripts are shown in Table 3.6.

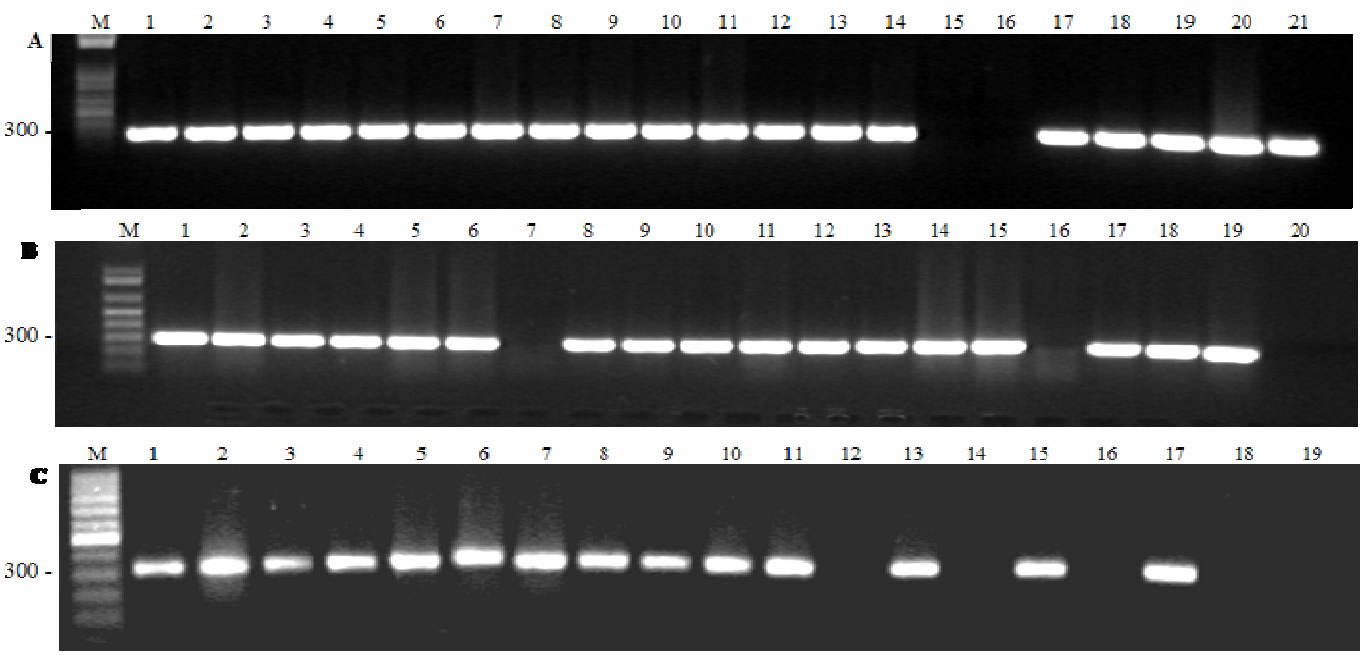

Figure 3.2. PCR using primer pair j5046 and j7069 for the detection of clones containing 3' end transcripts. Results of only representative clones are shown. The products were dominated by a 327 bp product in Tabanan/87 (Panel A), Pulukan/00 (Panel B) and Kal/00 (Panel C) strains of JDV. One clone from the Kal/00 strain produced a slightly larger product of 345 bp (Panel C, lane 6). Lane M, 100 bp (Panel C) and 1 Kb Plus (Panels A and B) DNA ladders (Invitrogen).

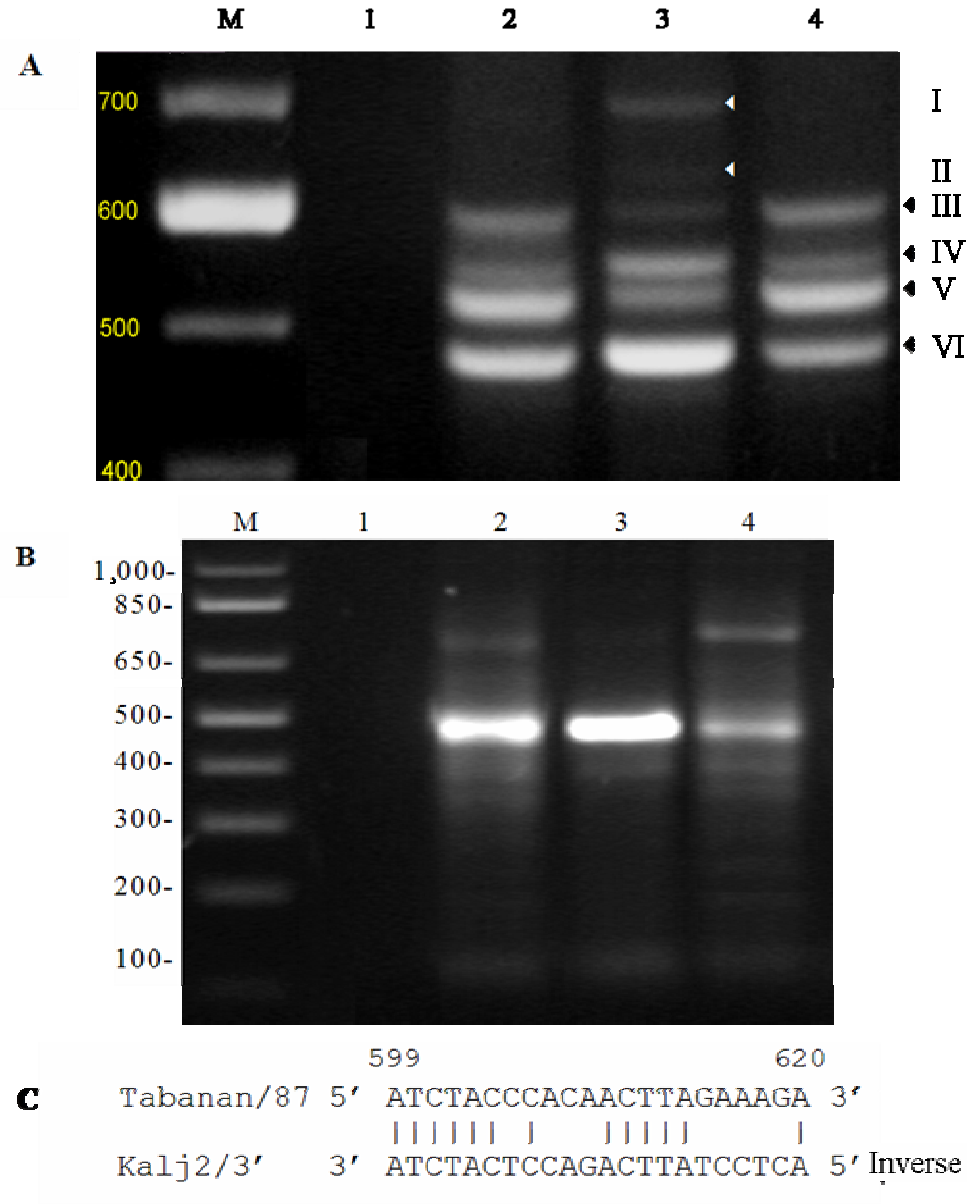

Figure 3.3. RT-PCR analysis of the 5' end of *tat* mRNA in tissues of Bali cattle experimentally infected with virus strains Tabanan/87 (Lane 2), Pulukan/00 (Lane 3) or Kal/00 (Lane 4); Lane 1, negative control; Lane M, 100 bp DNA marker (Invitrogen). Panel A. Amplification with j129 and junction primers for the predominant splicing junction j5335^j7033 (shown in Table 3.1) revealed *tat* mRNA of various sizes (types I–VI). The composition of each transcript is illustrated in Figure 3.5, Panel B. Panel B. When a 3' primer homologous to the second splicing junction found in the Kal/00 strain (Figure 3.2C, lane 6) was used a product was amplified from a different region of JDV. Sequence analysis identified the product as an unspliced RNA which was amplified as a result of the considerable sequence homology between the junction primer and the region of *gag* as shown in Panel C

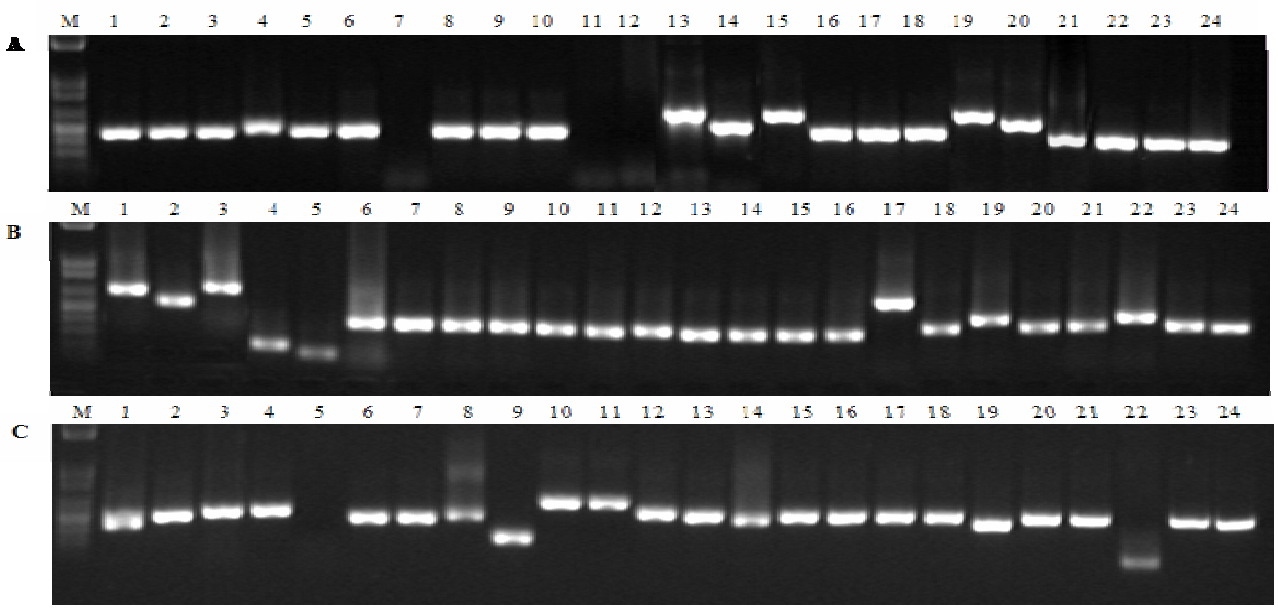

Figure 3.4. PCR screening using j129 and junction primers homologous to the 5335^7033 sequence for the detection of clones containing 5' end transcripts. Representative clones from Tabanan/87 (Panel A), Pulukan/00 (Panel B) and Kal/00 (Panel C) strains are shown. The products obtained were predominantly the smaller products of 490 bp (type VI), 515 bp (type V), 553 bp (type IV) and 583 bp (type III). The frequency of the larger 638 bp (type II) and 706 bp (type I) products (Panel B, lanes 1–3 and 17) detected only in the Pulukan/00 strain, was much less. Sequence analysis of representative clones revealed the presence and absence of non coding exons between the major splice site at nt 204 and *tat-1*, illustrated in Figure 3.5. The products shown in Panel B, lanes 4 and 5, and Panel C, lanes 9 and 22, were determined by sequence analysis to be not of JDV origin. Lane M, 100 bp (Panel C) and 1 Kb Plus (Panels A and B) DNA ladders (Invitrogen).

Table 3.6. Splice sites used to generate *tat* mRNA transcripts identified in Bali cattle experimentally infected with 3 JDV strains.

Nt position[1]	Sequence	Notes
204[1]	UGAG/GUGAGUAC	major SD
4081	UUAU/GUAAGUAU	non coding exon SD
4808	AGUG/GUGAGCCA	non coding exon SD
5268[2]	ACAG/AGAGCGGU	*tat* exon 1 SD[2]
5335[1]	CACG/GUGAGAUC	major *tat* exon 1 SD
4014	AAUGCUCGUAUACAG/GUAC	non coding exon SA
4663	UGAUGACACCCGCAG/GAAC	non coding exon SA
4894	GCUGGUAUCCGGUAG/GGAC	*tat* exon 1 SA
4939[1]	UGCAUUUUACAACAG/GGAA	*tat* exon 1 SA
6947[2]	UCCGAUACUUGGCAG/ACUU	*tat* exon 2 SA[2]
7033[1]	UAUUUUGCAUUUAAG/GUCC	major *tat* exon 2 SA

[1] Nt position previously predicted from sequence analysis by Chadwick *et al*. (1995).

[2] These splice sites were identified in only one transcript in animal infected with Kal/00 strain of JDV.

Comparison of nucleotide sequence of different strains

In addition to the 3 strains of JDV (Tabanan/87, Pulukan/00 and Kal/00) the samples analysed included material present in spleen from an animal with Jembrana disease in Kalimantan in 2003 (Kal/03), the same region from where the Kal/00 strain was obtained 3 years previously. Amplification of Kal/03 using primer pairs j4938 and j5338 for Tabanan/87and Pulukan/00 strains, and j4938 and jK5337 for Kal/00 and Kal/03 strains yielded bands of 400 bp (Figure 3.6). Products of similar size were also amplified from PBMCs of cattle experimentally infected with Tabanan/87, Pulukan/00 and Kal/00.

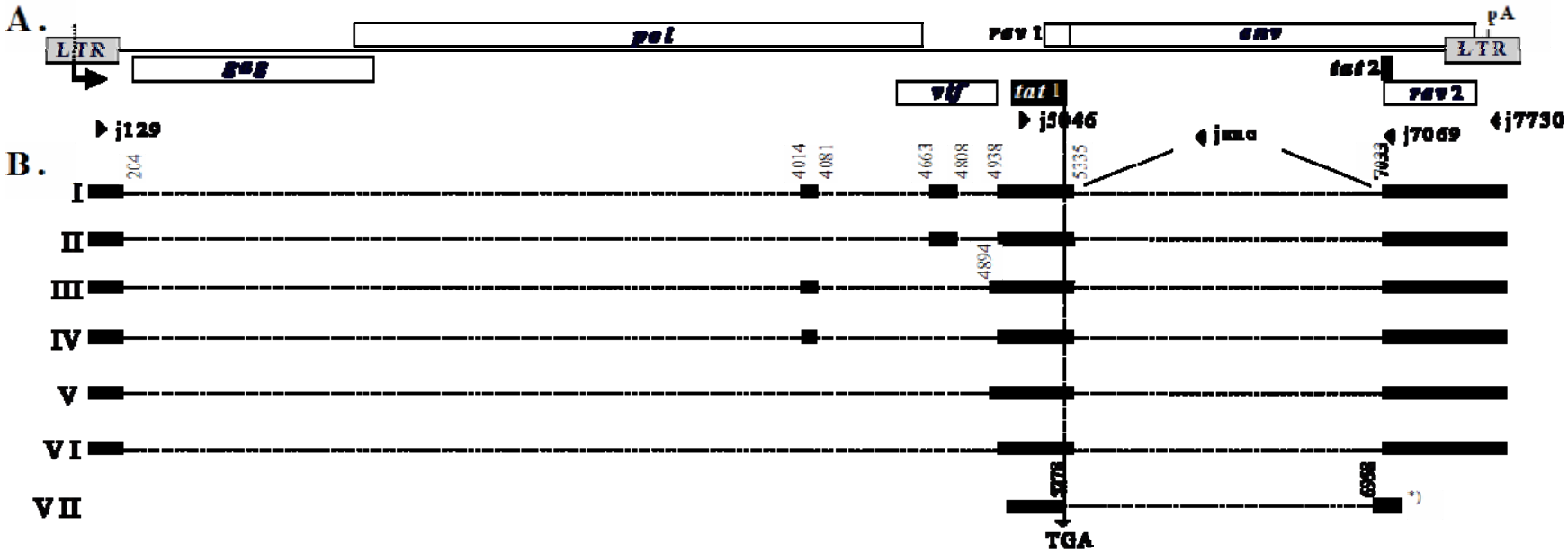

Figure 3.5. . Amplification strategy for the identification of JDV *tat* transcripts. (A) The JDV genome is flanked by LTRs where start (broken arrow) and termination (pA) sites of transcription are located; boxes represent the viral open reading frames including *tat*-1 and -2 (black filled). Arrow heads indicate the position and orientation of oligonucleotide primers used for cDNA detection (as shown in Table.3.1); "junc" refers to primers that were complementary to the sequence of splicing junction between the 2 *tat* exons. (B) Multiply-spliced transcripts (I-VI) detected which contained discrete exons (black boxes) by the removal of introns (horizontal broken line); the SD and SA nt positions are shown by the numbers at the top right and left of the exons, respectively. Splicing between *tat*-1 and *tat*-2 in transcript types I-VI was taken place 34 bases downstream the TGA stop codon at nt 5301 of *tat*-1 and nt 7033 of *tat*-2. A truncated *tat* transcript (type VII) detected in one transcript from Kal/00 contained a SD pstream of the end of *tat*-1 (nt 5278) and spliced to a SA in *env* (nt 6958).

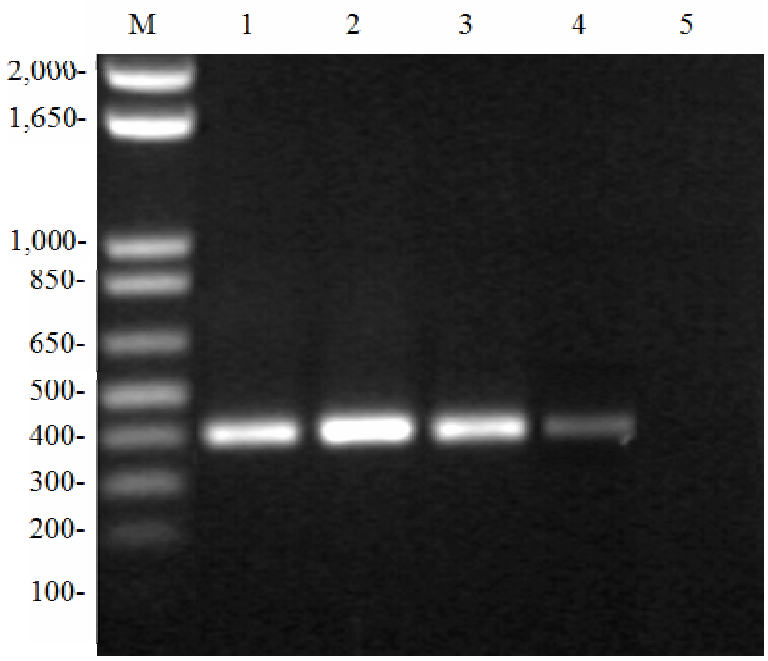

Figure 3.6. Amplification of *tat* exon 1 proviral DNA. Total DNA isolated from 4 spleen samples was subjected to PCR using primer pairs j4938 and j5338 for Tabanan/87 (Lane 1) and Pulukan/00 (Lane 2), and j4938 and jK5337 for Kal/00 (Lane 3) and Kal/03 (Lane 4). Lane M, 100 bp ladder. Products of the appropriate size were detected from all strains.

Analysis of nt sequences of the two *tat* exons identified variation in exon 1 and conservation in exon 2 (Figure 3.7). The *tat* exon 1 of all strains was 294 nt in size, corresponding to nt 5010–5303 of the genome; the sequence of Tabanan/87 was identical to that reported previously by Chadwick *et al* (1995b). The two Kalimantan isolates (Kal/00 and Kal/03) obtained from cattle during outbreaks in the same district but 3 years apart, were identical. Less than 5 % variation was detected between the Tabanan/87 and Pulukan/00 strains obtained from cattle in Bali 13 years apart. Nucleotide identity with reference to the Tabanan/87 strain was 89.1 % and 96.6 % for Kal/00 and Pulukan/00, respectively (Table 3.7). Nucleotide identity between Pulukan/00 and Kal/00 was 89.5 %. Alignment of the deduced Tat-1 amino acid sequences illustrated in Figure 3.8 showed identity of 83.5 % (Kal/00), 93.8 % (Pulukan/00) to the Tabanan/87 and 84.5 % between

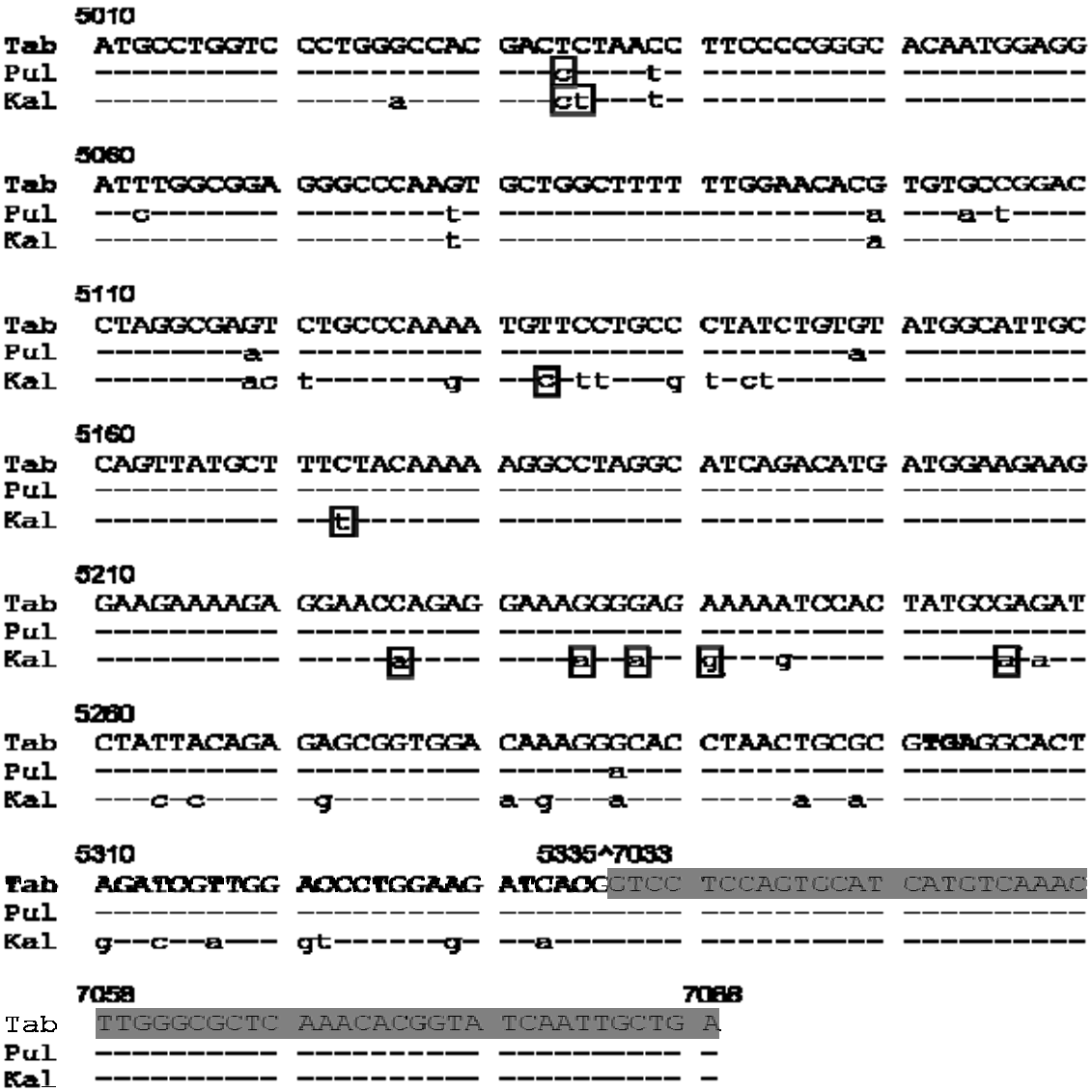

Figure 3.7. Nucleotide sequence alignment of the two *tat* coding exon from the spliced transcripts of 3 JDV strains Tabanan/87 (Tab), Pulukan/00 (Pul) and Kalimantan/00 (Kal). Dashes and lower case denote nt identity and substitution, respectively. Variation was evident in the first *tat* exon up to the junction (nt 5010–5335); some substitutions were silent (boxed). There was only limited variation between strains from the same geographical area. There was little variation in the nt sequence of exon 2 (shaded) in the 3 strains. ^ indicates the fusion of 2 discontiguous regions of the viral genome. The numbers showing the actual nt position in the genome are based on the sequence of the Tabanan/87 sequence (Chadwick *et al.*, 1995b).

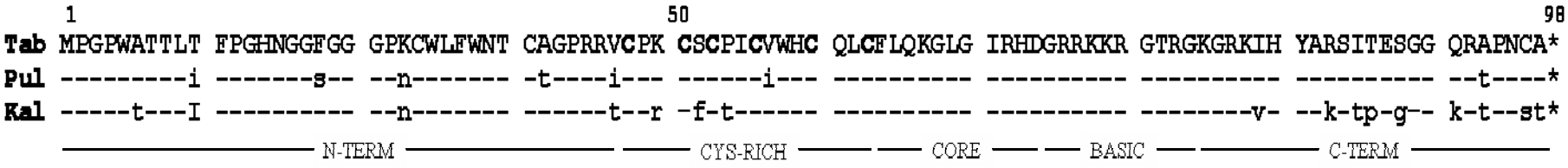

Figure 3.8. Alignment of Tat-1 amino acid sequence deduced from proviral DNA sequences. The alignment was generated and displayed by PileUp and Pretty programs, respectively. The sequence of Tabanan/87 is shown in capital letters. Dashes and lower case letters indicate amino acid identity and substitution, respectively. Variation was identified between the 3 JDV strains (Tab, Tabanan/87; Pul Pulukan/00; Kal, Kalimantan/00) , and the Kal strain was the most divergent. There was conservation in the cysteine residues (bold) in the Cys-rich region, and of all residues in the core and basic regions.

Pulukan/00 and Kal/00. There was a high degree of conservation in the cysteine residues in Cys-rich region, and identity in the core and basic regions.

Table 3.7. Percentage of nucleotide and amino acid identities between JDV strains.

Virus strain	Tab/87		Kal/00	
	nt	aa	nt	aa
Pul/00	96.6 %	93.8 %	89.5 %	84.5 %
Kal/00	89.1 %	83.5 %		

Discussion

While limited studies of the transcriptional events involved in the replication of BIV have been undertaken (Fong *et al.*, 1997; Liu *et al.*, 1992) there have been no such studies involving the related JDV. A major reason for this is the difficulty in gaining access to material for study: the virus has been recognised and detected in Indonesia only; the virus has not been successfully cultivated *in vitro* necessitating access to tissues of infected animals for analysis of transcriptional events. This current study has therefore provided novel information in relation to the transcription of *tat*, potentially important with regard to the relationship of Tat to the acute pathogenicity of JDV, and the development of Tat proteins as immunogens for the induction of a protective immunity against Jembrana disease.

Nucleotide sequence analysis of JDV *tat* cDNA clones showed that the *tat* gene was transcribed as at least 6 different mRNA transcripts predominated by types III–VI transcripts via multiple splicing events, a strategy typical of lentivirus transcription (Coffin *et al.*, 1997; Tang *et al.*, 1999). The mRNA transcripts differed in the SA sites utilised upstream of the first coding exon, and by the presence/absence of non-coding exons between the major SD downstream from the LTR and the SA at *tat*-1. Splicing between *tat*-1 and *tat*-2 was predominantly from the SD (nt 5335) downstream of *tat*-1 with the SA (nt 7032) of *tat*-2. The presence of an in-frame stop codon of *tat* exon 1 before the splicing junction

between *tat*-1 and *tat*-2 suggests *tat*-2 may not be translated. This splicing pattern was similar to BIV (Fong *et al.*, 1997; Liu *et al.*, 1992) wherein, despite the occurrence of mRNAs derived by the joining of both *tat* exons, only Tat103 encoded by *tat*-1 was detected (Fong *et al.*, 1997), and evidence of stop codon read-through was not indicated.

In the current study, the only material examined was from infections of the natural host during the acute phase disease of Jembrana disease. This may have affected the results obtained as in HIV-1, of which two Tat proteins of different size were detected. In the early stage of HIV-1replication where population of viral mRNAs is dominated by multiply spliced transcripts of ~2 kb encode for regulatory proteins, two-exon Tat was produced, whereas in the late stages one-exon Tat was translated from singly spliced transcripts of ~4 kb whose cytoplasmic transport is Rev dependent in contrast to multiply spliced transcripts that can be exported easily to the cytoplasm for protein synthesis (Malim *et al.*, 1988; Purcell & Martin, 1993). Sequence analysis of multiply spliced *tat* transcripts detected in this study suggested that a protein specified by *tat*-1, predicted to be 97 aa, was synthesised at least during the viraemic phase of Jembrana disease when there is maximal virus replication and plasma virus titres of 10^8 ID_{50}/mLor higher has been reported (Soeharsono *et al.*, 1990). These conclusions are supported by 2 additional observations. First, a protein band recognised by anti-Tat antibody was detected in PBMCs of experimentally infected cattle which size suggested as one-exon Tat (Presented in Chapter 4). Second, previous studies have demonstrated that the protein encoded from *tat*-1 only was fully functional and strong *trans*-activator (Chen *et al.*, 1999; 2000) suggesting *tat*-2 may not be essential for *trans*-activation. Whether or not a different pattern is operating prior to viraemia cannot be elucidated in this study as attempts to detect *tat* mRNA in PBMCs collected prior the febrile phase was unsuccessful. As lymphoreticular organs have been shown to be the main JDV target, lymphonode biopsies may provide a good source of transcripts.

While Tat encoded by *tat*-1 of JDV has been shown to generate sufficient *trans*-activation for efficient and high level replication, in HIV-1 the Tat encoded by the

spliced product of both *tat* exons is required to produce efficient upregulation (Bieniasz *et al.*, 1999a; Chen *et al.*, 1999; Fong *et al.*, 1997; Jeang *et al.*, 1999). The second coding exon of HIV-1 has been associated with increased viral infectivity, TAR-independent *trans*-activation of *env*, and cellular internalisation (Kameoka *et al.*, 2002; Kim & Panganiban, 1993; Ma & Nath, 1997; Tong-Starksen *et al.*, 1993; Verhoef *et al.*, 1998). Further study is required to examine the possible expression and function, if any, of the second *tat* exon JDV Tat.

Although it was detected only in one occasion on Kal/00 strain and appeared to be unusual, a shorter transcript type VII (Figure 3.5, Panel B) utilised a SD upstream the 3' end of *tat* exon 1 and was spliced to a SA in *env*. The splicing site(s) between the common transcription start site in the LTR and *tat*-1 exon; however, were unsuccessfully detected due to the high similarity between the junction primer and an area in the *gag* region. This mRNA transcript might encode a truncated Tat protein 10 aa less than the predicted normal Tat of 97 aa, and possibly fused with the protein encoded by the 5' end of *env* in frame with Rev. Patterns smilar to this was also detected in HIV-1 and EIAV which resulted in new chimeric proteins, such as Tev and Ttm (Benko *et al.*, 1990; Salfeld *et al.*, 1990; Solomin *et al.*, 1990).

Mutation was evident in *tat*-1 but not *tat*-2 of the three JDV strains examined, and dominated by G to A substitution consistent with that described for most of the lentiviruses (Borman *et al.*, 1995; Fitzgibbon *et al.*, 1993; Perry *et al.*, 1992; Vartanian *et al.*, 1994; Wain-Hobson *et al.*, 1995). There was evidence of geographical variation in *tat* but further study of other geographically different samples is needed to clarify this observation, and to determine the significance of the variation. Jembrana disease has been dispersed throughout some islands of Indonesia over a more than 30 year period, apparently originating in Bali in 1964, then occurring in East Java, then Lampung Province in Sumatra, then in the mid 1990s in West Sumatra, then also in the mid 1990s it was detected in South Kalimantan. While the origin of the South Kalimantan strain is unknown, the limited sequence data obtained in this study suggests that the Bali and Kalimantan strains were different. Direct transmission from Bali to Kalimantan is

unlikely due to quarantine restrictions on the movement of cattle between these provinces. Possibly the virus may have been introduced into Kalimantan from Sumatra, from strains originally transmitted to Lampung province in Sumatra in the 1970s, and then subsequently to Kalimantan in the late 1990s. Further sequence analysis of strains from all these areas, which is in progress, may help to determine the origin of the various strains in different islands of Indonesia.

The nucleotide variation of JDV *tat*-1 was reflected in a similar variation of the amino acid sequence (Table 3.7). However, it was observed in Figure 3.8 that the core and basic regions of the putative protein encoded by *tat*-1 exhibited only limited variation and the cysteine residues in the Cys-rich region were completely conserved similar to that of HIV-1 (Chakrabarti *et al.*, 2001; Gregoire & Loret, 1996; Husain *et al.*, 2001; Reza *et al.*, 2003; Scriba *et al.*, 2002). Nucleotide sequence of the core region was highly conserved among the three strains, while considerable substitutions in the basic region of Kalimantan strain were mostly synonimus (Figure 3.7). The limited variation in *tat* would be consistent with an important role of the Tat protein in virus replication, especially in the regions that are critical in RNA-binding and transcription activation (Carvalho & Derse, 1991; Cullen & Garrett, 1992; Frankel, 1992; Jones & Peterlin, 1994; Jones, 1997; Ruben *et al.*, 1989). Substitution of any cys residues except C31 has an adverse effect on *trans*-activation activity of Tat, while conservative substitution C31G or C31S has a minimal consequence (Jeang *et al.*, 1999).

In conclusion, the results suggest that a Tat protein specified by coding exon 1 of *tat* was the predominant if not the only Tat protein produced during the acute phase of Jembrana disease, and was conserved in different strains of JDV strains. The critical dependence of JDV replication on Tat, and its high conservation within strains from widely divergent areas of Indonesia, suggests it is a potential target for the control of JDV infection and warrants further study as a possible vaccine candidate. The detection of the Tat protein *in vivo* and production of this protein using recombinant DNA technology for the purpose of immunisation are described in the Chapter 4.

Chapter 4

Production of recombinant proteins and immunogenicity examination *in vivo*

Summary

The expression and purification of recombinant JDV Tat was investigated utilising different vectors in bacterial and eukaryotic expression systems. The *tat* exon 1 DNA was cloned in frame to glutathione S-transferase (GST) or hexa-histidine (His_6) genes, for expression in *E. coli* under the regulatable *tac* promoter. Tat was also expressed constitutively as a His_6 fusion protein in mammalian cells driven by the CMV promoter. Tat was expressed in *E. coli* mostly within inclusion bodies either as a ~38-kDa GST fusion protein (GST-Tat) or a ~17-kDa His-Tat fusion protein. Purification of GST-Tat was achieved by extensive detergent washing or urea-mediated solubilisation of inclusion bodies, while His-Tat was purified using nickel affinity chromatography. The immunogenicity of GST-Tat in sheep and Bali cattle was demonstrated by induction of a specific antibody response in inoculated animals. The specific antibody response was determined by Western immunoblotting utilising recombinant Tat produced in the eukaryotic expression system, so avoiding cross-reactivity with *E. coli* proteins. Antibodies to Tat was also detected in cattle recovered from natural JDV infection, and in cattle immunised with a whole virus vaccine derived from spleen tissue of JDV-infected cattle although the frequency and level of antibodies to Tat in these cattle were less than those to the JDV capsid protein. Affinity purified sheep anti-Tat was able to detect the native Tat protein with an apparent molecular size of ~14 kDa in PBMCs of JDV-infected Bali cattle.

Introduction

Within Indonesia where Jembrana disease is endemic, an inactivated vaccine is used for the control of the disease. Although there are no published reports on the efficacy of this procedure, experimentally the vaccine has been shown to ameliorate the clinical signs of disease following challenge with virulent virus, and there is considerable anecdotal evidence that it is effective and has been favourably received by cattle owners in these areas (Dr Nining Hartaningsih, Personal Communication). This Jembrana disease vaccine is prepared from spleen tissue of experimentally infected cattle, collected during the acute febrile phase of the induced disease when there is a high titre of infectious virus in spleen tissue (Hartaningsih *et al*., 2001). There are a number of safety concerns regarding this vaccine: the virus in the vaccine is inactivated with detergent and this would not inactivate non-enveloped adventitious virus present in the donor animal; there is no method of standardisation of the quality of the vaccine in different batches; it is expensive to prepare, requiring the use of donor animals from disease-free areas; the current method of production of the vaccine is not applicable for commercial production techniques.

In an attempt to develop a safe and effective JDV vaccine, the studies on the potential of using JDV Tat protein, produced by recombinant DNA technology, as an alternative vaccine, was explored. Previous studies toward the control of HIV-1 infection have considered Tat an attractive target immunogen for several reasons: first, Tat protein is produced early during the virus infection (Wu & Marsh, 2001); second, it is indispensable for efficient virus replication (Arya *et al.*, 1985; Dayton *et al.*, 1986); third, Tat is immunogenic and Tat antibody correlated with low plasma viral load and delayed disease progression in HIV infected subjects (Re *et al.*, 2001a; Reiss *et al.*, 1990; Zagury *et al.*, 1998b). Vaccination experiments in animal models using active or inactive Tat have been shown to induce specific immune responses and confer protection against disease progression (Agwale *et al.*, 2002; Barillari *et al.*, 1999; Pauza *et al.*, 2000). Antibodies from human volunteers immunised with Tat toxoid presented high

antibody titres to Tat (1998; Gringeri *et al.*, 1999) and inhibited Tat-mediated *trans*-activation (Noonan *et al.*, 2003). It was hypothesised that a similar approach would be applicable for Jembrana disease, and immunisation with recombinant Tat could overcome problems concerning the viral antigen production.

In Chapter 3, mRNA transcripts potentially responsible for Tat protein production in infected cattle were identified. These studies suggested that of the two *tat* coding exons of JDV, Tat of 97 aa encoded by exon 1 only is translated during the peak viraemia of the disease, in agreement with a previous report that the first *tat* exon (tat1) is sufficient to generate a functional Tat (Chen *et al.*, 1999). In the studies reported in this Chapter, therefore, we considered recombinant technology as a mean to produce JDV Tat from *tat*1, and to demonstrate the immunogenicity and antigenicity of the expressed products. For the development of a potential immunogen that might be appropriate as a vaccine, attempts were made to express Tat in *E. coli* as either a glutathione-S-transferase (GST) and hexahistidine (His_6) fusion protein, using plasmids pGEX-6P and pTrcHis vectors, respectively. To increase the specificity of Western immunoblotting procedures for the detection of anti-Tat, an attempt was made to express JDV Tat in mammalian cells. The immunogenicity of the purified GST-Tat fusion protein was investigated in sheep in Australia, and then Bali cattle in Indonesia. For additional evidence of the immunogenicity of Tat in Bali cattle, the occurrence of anti-Tat antibody in JDV-vaccinated and recovered cattle was also investigated.

Materials and methods

Host cells, media and growth conditions.

The *E. coli* Top10F' [F´{*lac*I^q Tn*10*(Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] (Invitrogen) was used in this study. Luria-Bertani broth supplemented with 100 µg/mLampicillin (LBA) was used for growth of bacteria at

37°C with agitation. For protein expression by bacteria, deficient M9 (Sambrook *et al.*, 1989) and LB media, both containing 2 % glucose, 100 µg/mLampicillin and 50 µg/mLtetracycline, were used.

COS7 (African green monkey kidney) cell line was grown in 25 or 75 cm^2plastic flasks (Nalgene Nunc International) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % foetal bovine serum, 100 U/mLpenicillin, 100 µg/mLstreptomycin, and 2mM L-glutamine at 37°C in a 7 % CO_2 atmosphere. When monolayers were confluent, cells were passaged by trypsinisation (0.25 % Bacto-trypsin; 1 mM EDTA; Hank's balanced salt solution) using a split ratio of 1:3, or maintained in DMEM with 2 % calf serum.

Cloning vectors

The TA-cloning vector pCR2.1 (Invitrogen) was used to clone PCR amplification products, and served as a holding vector for these products. For bacterial expression studies, JDV *tat*1 was cloned in frame with glutathione-S-transferase (GST) in plasmids pGEX-6P-1 (Amersham Biosciences Biotech,) and a hexahistidine gene in plasmid pTrcHis A (Invitrogen). These 2 vectors drive expression of heterologous gene products from a strong and regulatable *tac* promoter in *E. coli*. For mammalian expression, pcDNA3.1/His C (Invitrogen) was used. This plasmid contains a SV40 origin of replication, which enables it to replicate episomally in SV40 T antigen expressing cells, such as COS7 (Gluzman, 1981; Kaufman, 1990; Makrides, 1999). The neo^r under SV40 promoter conveys resistance to G-418 sulphate antibiotic (Southern & Berg, 1982). The schematic diagrams of these expression vectors were depicted in Figure 4.1.

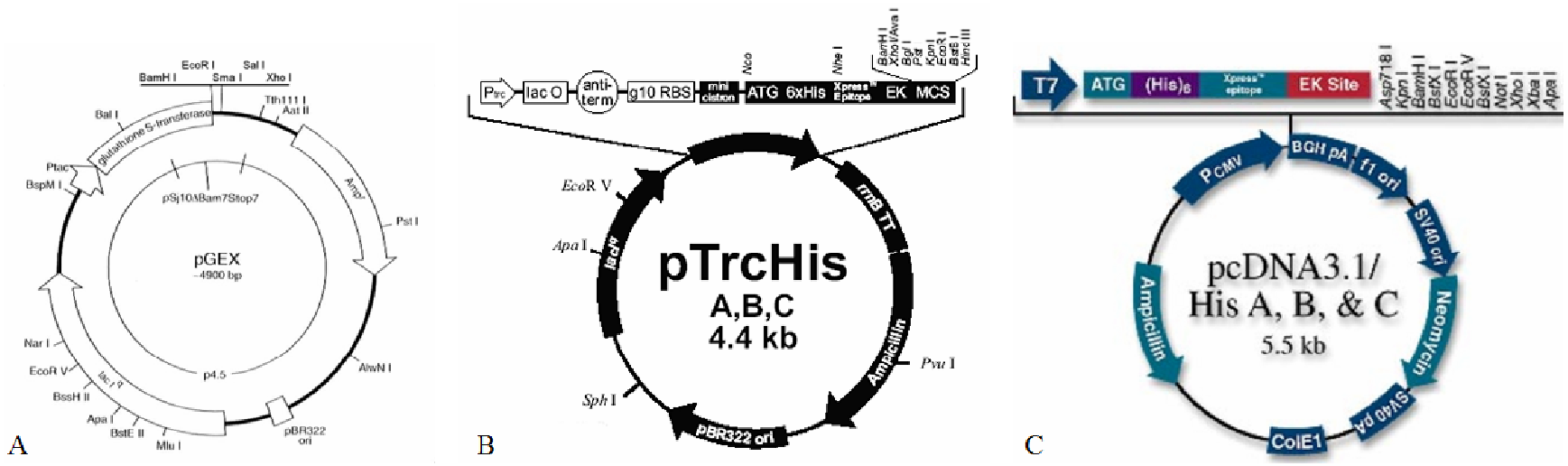

Figure 4.1. Map of the expression vectors used in this study. JDV *tat* DNA fragment was cloned into *Bam*H1/*Eco*R1 multiple cloning sites (MCS) of the vectors in frame with glutathione S-transferase (GST) or $(His)_6$ N-terminal fusion partners in pGEX (A, Amersham) or pTrcHis and pcDNA3.1/His (B and C, Invitrogen), respectively.

Oligonucleotides

The oligonucleotide primers shown in Table 4.1 were used during the course of these studies. Primers were purchased from Invitrogen and stocked as 200 µM solutions in sterile double-distilled water.

Table 4.1. Primers used for amplification and sequencing of JDV Tat constructs in different plasmid vectors.

Primer	Sequence (5' to 3')	Genome	Nt. Position [1)]
M13/F	GTAAAACGACGGCCAG	pCR2.1	205 – 221
M13/R	CAGGAAACAGCTATGAC'	pCR2.1	404 – 389
pGEX/5'	GGGCTGGCAAGCCACGTTTGGTG	pGEX-6P	869 – 891
pGEX/3'	CCGGGAGCTGCATGTGTCAGAGG	pGEX-6P	1056 – 1034
pTRC/5'	GAGGTATATATTAATGTATCG	pTrcHis	370 – 390
pTRC/3'	GATTTAATCTGTATCAGG	pTrcHis	614 – 597
pcDNA/T7	TAATACGACTCACTATAGGG	pcDNA3.1	863 – 882
pcDNA/BGH	TAGAAGGCACAGTCGAGG	pcDNA3.1	1122 – 1105
jTat4990	ATCAACCGGatccCCAGATATGCCTG	JDV	4990 – 5016
jTat5328	TTCCAGGGTCCAACGATCTAGTGCCT	JDV	5328 – 5303

[1]The nt numbering system adopted was according to GenBank accession No. U21603.

Restriction endonuclease digestion

Restriction enzyme digestions were performed by incubating double-stranded DNA with an appropriate amount of endonucleases in their respective buffers as recommended by the supplier (NEB), at the optimal temperature for that specific enzyme, which was typically 37°C. Standard digestions include 2–10 U enzyme per microgram of DNA in 50 µl reaction. The reactions were usually incubated for 1–3 hr to ensure complete digestion which was subsequently checked by gel electrophoresis.

Construction of expression vectors

DNA fragment containing the first coding exon of JDV *tat* (tat1) encompassing nt 5010 to 5303 was generated by PCR from proviral DNA of the Tabanan/87-strain present in infected spleen tissue (Chapter 3) using synthetic oligonucleotides jTat4990 5'-ATCAACCGGatccCCAGATATGCCTG-3' (nt 4990–5016) and jTat5328 5'-TTCCAGGGTCCAACGATCTAGTGCCT-3' (nt 5328–5303). To facilitate further cloning, the forward primer jTat/5' was mutated (lowercase) to contain a *Bam*H1 site (underlined) before the start codon.

PCR was performed using the Expand™ high fidelity PCR system (Roche) with the following cycling conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation (30 sec at 95°C), annealing (30 sec at 65°C) and elongation (1 min at 72°C), followed by a final elongation step at 72°C for 7 min. The predicted PCR product of 339 bp was TA-cloned. By TA-cloning, the correctly amplified *tat* fragment, confirmed by sequencing, was then available for further cloning experiments, avoiding repeated amplification and introduction of sequence errors by polymerases. DNA sequencing was performed as described in Chapter 3.

The JDV *tat* fragment was released from pCR/*tat* by double digestion with *Bam*HI and *Eco*RI restriction enzymes (NEB). Following agarose gel electrophoresis, the fragment was excised and purified using a QIAquick Gel Extraction Kit (Qiagen). The fragment was then ligated into the corresponding site of digested plasmid vectors to generate pGEX/*tat*, pTrc/*tat* and pcDNA/*tat*. Recombinant plasmids were screened by PCR and further confirmed by nucleotide sequencing using the respective vector-specific primers (Table 4.1).

Expression of recombinant proteins

E. coli *transformation and induction*

E. coli TOP 10F' cells were transformed with pGEX/*tat* or pTrc/*tat* by heat-shock treatment as previously described (Chapter 3). A single colony of each transformant was inoculated in 5 mLLB broth supplemented with 100

μg/mLampicillin, 50 μg/mLtetracycline and 2 % glucose as a starter culture and incubated at 37°C with agitation on a shaker (250 rpm) overnight. The overnight culture was diluted 1:20 into 200 mLmedia in a 1,000 mLflask, and continuously agitated at 250 rpm for 1–2 h at 37°C until the OD_{600} measured 0.5. For induction of expression, IPTG was added to a final concentration of 0.1mM for GST and 0.5 mM for His_6 fusion constructs, and the incubation continued for an additional 1.5 h. Comprehensive expression tests in small scale of 5 mLwere performed earlier to determine the optimum conditions with respect to IPTG concentration (0.1–1 mM), length (1–4 h) and temperature (30 and 37°C) of induction, as well as the use of enrich and deficient media for high expression levels of soluble protein.

Transfection and protein expression of eukaryotic cells

The day before transfection, cells were seeded at 2 x 10^5 cells/cm^2 in 25 cm^2 flasks, which developed to 80–90 % confluency the next day. Following brief washing with DMEM, the cells were exposed for 3 h to a mixture containing 10 μg of pcDNA/*tat* plasmid DNA and 50 μl of Lipofectamine 2000 (Invitrogen) in 1 mLserum free DMEM. After incubation for 3 h at 37°C, the transfection medium was replaced with growth medium and the cells were incubated for further 72 h. The transfected cells were selected in growth medium containing 500 mg/mLG-418 sulphate (GIBCO) which provides substrate for the selection marker aminoglycoside phosphotransferase (neo^r). The resistant cells were serially diluted and propagated to produce an adequate amount of Tat-expressing cells. Cells transfected with control pcDNA3.1 were used as a negative control.

The transfection efficiency was determined by transfecting triplicate cultures of COS7 cells in 24-well plates with pcDNA3.1/His/*lacZ* with a range of different DNA concentrations and volumes of lipofectant. Transfected cells were washed with PBS, fixed with 0.05 % glutaraldehyde in PBS for 10 min, and stained with X-gal and then incubated at 37°C for 2 h. Transfection efficiency was determined by examining the cells with an inverted microscope and determining the percentage of galactosidase positive cells relative to the total cells.

Purification of GST-fusion protein

Purification of soluble fraction

The *E. coli* cells from a 200 mLIPTG-induced culture were centrifuged (4,000 *g*, 4°C, 10 min) following the addition of phenylmethylsulfonyl fluoride (PMSF) to a final 1 mM concentration. The purification of soluble fusion was performed according to the GST gene fusion system protocol (Amersham Bioscience). Briefly, the pellet was resuspended in 4 mLice-cold phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3) with 1 mM PMSF, 1 mM EDTA, 5 mM 1,4-Dithiothreitol (DTT), 10 U/mLDNase I (Sigma) and 1 mg/mLlysozyme (Sigma)) and kept on ice for 30 min. Cell extracts were prepared by several cycles of freezing in liquid N_2 and thawing in a 42°C water bath until the solution was no longer viscous, and Triton-X was added to a final concentration of 2 %. After incubation for 30 min on a rocking platform, the lysate was centrifuged (20,000 *g*, 30 min, 4°C) and the supernatant transferred to a chilled fresh tube. To each 4 mLof prepared cell extract was added 400 µl of 50 % slurry of Glutathione Sepharose 4B (Sigma) previously equilibrated with PBS. The suspension was gently mixed at 4°C for 2 h on a rotating wheel. The Sepharose beads were pelleted by centrifugation (500 *g*, 5 min) and the supernatant carefully removed. The pelleted matrix was mixed with 10 mLof cold PBS for 5 min and centrifuged (500 *g*, 5 min); this washing step was repeated twice. The bound fusion protein was then eluted by the addition of 500 µl of glutathione elution buffer (20 mM reduced-glutathione; 50 mM Tris-HCl, pH 8.0). The tube was gently agitated by gentle inversion during the process (30 min, 4°C). Following centrifugation (500 *g*, 10 min) to sediment the beads, the eluant was removed and stored at –80°C until needed.

Purification of inclusion bodies

Cells and cellular debris from 200 mLcultures were washed repeatedly in 10 mLvolumes of wash buffer (2 % Triton-X100, 50 mM Na_2HPO_4, 300 mM NaCl, 2 mM EDTA, 10 % glycerol, pH 8.0) by centrifugation (13,000 *g*, 15 min, 4°C). The washing process was repeated until the supernatant was clear (~6 cycles). The

washed inclusion bodies were then solubilised in 4 mLof 6 M guanidium HCl (Gu-HCl) or 8 M urea, 50 mM Na_2HPO_4, 20 % glycerol, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 8.0) and stirred for at least 5 h at room temperature, then centrifuged as above. The supernatant containing solubilised inclusion bodies was stored at –80°C until further use.

For injection into sheep or cattle, the solubilised inclusion bodies were first dialysed against PBS at 4°C for 24 h with 3 changes of PBS. The solution was stirred at low speed during dialysis.

Purification of His-tagged proteins

High affinity of His-tagged proteins to a chelating resin charged with metal ions such as Ni^{2+}, Cu^{2+} or Zn^{2+}, has been used for purification from proteins which have lower, or no, affinity for the resin (Hochuli *et al.*, 1987; Porath *et al.*, 1975).

The advantage of histidine tagging is that it can be employed also under denaturing conditions (Holzinger *et al.*, 1996).

In this experiment, purification of His-Tat proteins was conducted under denaturing condition with a QIAexpressionist® System Protein Purification kit (Qiagen) as described by the manufacturer. Briefly, following 1.5 h of IPTG induction, PMSF was added to final concentration of 1 mM and the culture was centrifuged (4,000 *g*, 4°C, 10 min). The pellet was resuspended and stirred for 30 min on ice in 5 mLBuffer B (8 M urea, 100 mM NaH_2PO_4, 300 mM NaCl, 10 % glycerol, 1 % Triton-X100, 10 mM β-mercaptoethanol, pH.8.5) to lyse the cells. Cell debris was removed by centrifugation (20,000 *g*, 30 min, 4°C) and 0.5 mLof 50 % Ni-NTA slurry (Qiagen) was added to 5 mLof the supernatant and incubated for 1 h on a rotary wheel at room temperature. The mixture was centrifuged and washed 3 times with buffer C (same as Buffer B but pH. 6.5). The bound His-Tat was eluted with 0.4 mLbuffer D (same as Buffer B but pH. 5.8), followed by buffer E (same as Buffer B but pH 4.5). All fractions were collected and stored at –80°C until further analysis was conducted.

For His-Tat protein produced in eukaryotic system, purification was done under mild denaturing condition with the following modification. Five 25 cm^2 flasks of stably transfected COS7 cells were washed twice with ice cold PBS and lysed in 1 mLcold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 % glycerol, 10 mM imidazole, 0.1 % SDS and EDTA-free protease inhibitor cocktail [Sigma]) for 30 minutes at 4°C. The soluble protein-containing supernatant was separated by centrifugation (14,000 *g*, 15 min, 4°C) and 250 µl of 50 % Ni-NTA slurry equilibrated with lysis buffer was added to 5 mLof the supernatant. The process was continued as for the prokaryotic system, except wash buffer (lysis buffer with 20 mM imidazole) replaced Buffer C, and elution buffer (lysis buffer with 300 mM imidazole) replaced Buffer D.

Analysis of recombinant proteins

Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently visualised by Coomasie brilliant blue (CBB) or silver staining. The identity of the protein bands was confirmed by Western immunoblotting. The yield of the purified protein was estimated densitomerically.

SDS-PAGE

Proteins were analysed by SDS-PAGE on discontinuous gels (Laemmli, 1970). One dimensional gel electrophoresis was performed in a Mini-Protean 3 electrophoresis unit (BioRad) under reducing condition using a 15 % polyacrylamide gel with a 4 % stacking gel. The molecular mass standards were purchased from BioRad and Pharmacia. Immediately before electrophoreses, samples were boiled for 5 min in sample reducing buffer (0.5 M Tris-HCl, pH 6.8, 2 % SDS, 0.1 % bromophenol blue, 10 % glycerol, 5 % β-mercaptoethanol). The proteins were resolved at 150 V for 1.5–2 h at room temperature in running buffer containing 2.9 g/L Tris base, 14.4 g/L glycine, and 1g/L SDS.

Following electrophoresis, the protein bands were visualised in Coomassie brilliant blue (0.1 % CBB [BioRad], 40 % methanol, 10 % acetic acid) for at least

2 h at 37°C or overnight at room temperature. The gel was destained for about 2 h in solution containing 10 % acetic acid and 40 % methanol.

For the detection of eukaryotic recombinant Tat, the bands were visualised on the gel with silver stain (BioRad) according to the procedure recommended by the manufacturer. Briefly, gels were fixed for 10 min, washed with 50 % ethanol 3 times, then washed 3 times in water, placed in a silver staining solution for 20 min, washed in water for 2 min, then placed in a developing solution until bands appeared. The gel was finally treated with "stop" solution and washed with 50 % ethanol.

Western immunoblotting

The separated proteins were transferred from gels onto Hybond^{TM} nitrocellulose membranes (BioRad,) in a Mini Trans-Blot Module (BioRad). Transfer was performed in 25 mM Tris-HCl, 192 mM glycine, 20 % methanol for 2 h at 200 mA or overnight at 30V. The membrane was blocked with TBST buffer containing 1 % gelatin for 1 h at room temperature, and the blotted proteins were probed with primary antibodies for 1 h at room temperature or overnight at 4°C. After washing 4 times with TBST, the membrane was incubated with corresponding secondary antibodies for 1 h at room temperature. Following washing, the reaction was visualised using freshly made colour development reagent (BioRad) as described by the manufacturer. After the colour developed, the reaction was stopped by rinsing the membrane in water.

Primary antibodies to detect GST-Tat or His-Tat fusion proteins were goat anti-GST (Amersham Bioscience) and mouse anti-His_6 (Sigma), respectively. Both were used at 1:2,000 dilutions in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % Tween-20). The secondary antibody (1:3,000) was either horseradish peroxidase (HRP) conjugated anti-sheep, anti-mouse (ICN), anti-goat (Amersham Bioscience) or anti-bovine (ICN) immunoglobulin, as appropriate.

Concentration, molecular mass and pI estimation

The CBB-stained gel image was captured using ProXPRESS™ ProFinder (PerkinElmer) followed by volume analysis with Proteome 1D Analyzer V1.10 (PerkinElmer). The concentration was calculated by comparison of densitometric scans with known amounts of bovine serum albumin, BSA and lysozyme (Sigma) run on the same gel. Molecular mass and pI of proteins was calculated with ProtParam tool from the ExPASy molecular biology server accessible at http://au.expasy.org.

Production of Tat-specific antiserum

Polyclonal antibody was induced in sheep by injection of partially purified GST-Tat inclusion bodies (above). About 2 mg of GST-Tat was emulsified in incomplete Freund's adjuvant and subcutaneously injected into a sheep. Two booster injections were given intramuscularly at week 3 and 5 with the same preparations. Serum samples were obtained before injection and 2 weeks after each injection, and stored at –20°C for further analysis.

Due to the impurity of the Tat antigen used to produce specific antibodies, the serum was affinity purified by adsorption of antibody onto GST-Tat and then by removal of non-specific antibody by absorption against GST and bacterial proteins. In brief, GST-Tat expressing *E. coli* lysates were subjected to preparative SDS-PAGE and blotted onto nitrocellulose membranes. The membrane was stained with Ponceau solution (Sigma) and the GST-Tat band was excised and destained by washing with water. The GST-Tat band from 4 gels was incubated with 8 mLof a 1:5 dilution of the sheep serum in PBS, with gentle shaking at 4°C overnight. The nitrocellulose strips were then extensively washed with PBS. The adsorbed antibodies were eluted from the strips by placing them in 4 mL0.1 M glycine-HCl (pH 2.8) for 10 min and then immediately neutralised with 1 M Tris and subsequently brought up to 20 mLwith PBS. Non-specific antibodies were absorbed against total GST cell lysate blotted membranes overnight. The remaining antibody solution was dialysed against

PBS for 24 h at 4°C and 10 x concentrated by microfiltration (PAL). The final antibody preparation was used for immunoblotting at a 1:200 dilution.

Detection of native Tat protein

Native Tat proteins were detected by WB using an enhanced chemiluminescent detection system (ECL™, Amersham Biotech). Peripheral blood mononuclear cells (PBMCs) and spleen tissues were collected from experimentally infected cattle as described in Chapter 3. Proteins were prepared by adding sample reducing buffer (above) directly to pelleted PBMCs or 50 % spleen homogenates in PBS. Samples were subjected to SDS-PAGE using 17.5 % gel and blotted onto Hybond™ ECL™ membrane. Non-specific binding was blocked with 5 % Blocking reagent in TBST. Proteins were detected by subsequent incubation with the affinity-purified polyclonal sheep anti-Tat at 1:200 dilution in blocking solution followed by a 1:5,000 dilution of secondary horseradish peroxidase conjugated antibody (ICN) for 2 h and 30 min, respectively. After extensive rinsing in TBST the membrane was placed on Saran wrap with protein site up and a fresh mixture of detection solution ECL 1 and ECL 2 (3 mLeach) was poured. After 1 min, the membrane was drained, wrapped and exposed to Hyperfilm™ ECL™ for 10–15 s in a film cassette. The film was processed with a film developer machine (Fuji). All the development steps were done in a dark room.

Immunogenicity of GST-Tat in Bali cattle

Sera were obtained from 4 Bali cattle that had been injected 3 times (in Indonesia) with a preparation of GST-Tat emulsified in incomplete Freund's adjuvant. The injections were repeated twice at 2-week interval. Sera were collected on day 0, 14, 28 and 42 after the initial injection. Immune reactivity of the sera against GST and eukaryotic His-Tat by Western blotting was investigated.

Detection of antibody to Tat in Bali cattle

One hundred and forty three serum samples were obtained from the Disease Investigation Centre, Region VI, Bali, Indonesia. Three sera were from cattle recovered from experimental infection (convalescent sera collected 1 month post infection), 81 were from cattle vaccinated with the crude spleen vaccine in Bali, 26 were from similarly vaccinated cattle in South Kalimantan, 15 were from uninfected cattle in the Jembrana disease-free provinces Nusa Penida (5 sera) and Sulawesi (10 sera), and 10 sera with unknown vaccination history were collected from Bali. Convalescent sera were collected monthly in 6-month period since the day of virus inoculation. Sera from the vaccinated animals were collected 1 month after 3 injections at monthly intervals of the inactivated vaccine. Some of the sera had been tested with an ELISA using whole virus as the antigen (Hartaningsih *et al.*, 1994).

Recombinant His-Tat_{mam} produced in mammalian cells and capsid protein fused to GST, GST-CA (Burkala *et al.*, 1998) were used as antigens in western immunoblots. GST-CA was produced in bacteria and affinity purified on glutathion sepharose as described earlier. The purified proteins were resolved by 15 % SDS-PAGE and transferred to nitrocellulose membranes, and subsequently cut into strips. The antigen strips were incubated with 1:20 dilution of bovine sera, followed by anti-bovine conjugate (ICN) at 1:2000 and developed with Biorad colour development reagent.

Results

Construction of JDV Tat expression vectors

The first coding exon of JDV *tat* was amplified from Tabanan/87 proviral DNA by PCR and cloned into pCR2.1 (Invitrogen), followed by subcloning in the correct orientation into the *Bam*H1 and *Eco*R1 sites of pGEX and pTrc/His vectors for expression in *E. coli* (Figure 4.1A and B). In addition, pcDNA3.1/His vector (Figure 4.1 C) was used to express Tat-1 in mammalian cells under the human

cytomegalovirus (CMV) immediate-early promoter (P_{CMV}) control. Transformants containing Tat constructs were screened by PCR using primer combinations specific to vector and *tat* sequences (Table 4.1) which produced amplicons with the expected sizes (Table 4.2) are shown in Figure 4.2. That the inserts were in the correct orientation and in-frame was confirmed by nucleotide sequencing.

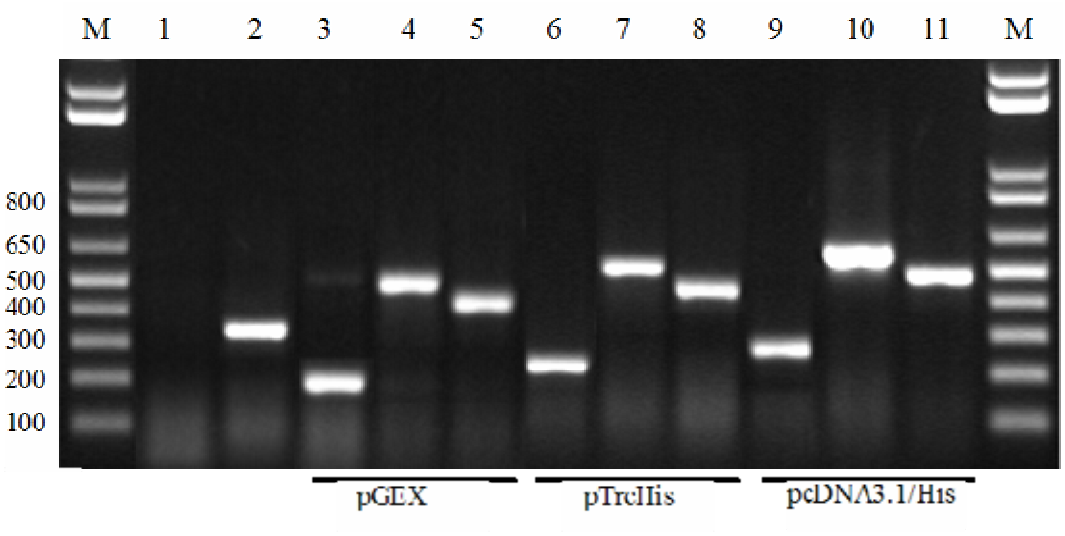

Figure 4.2. Insert amplification and analysis of transformants. JDV *tat* DNA was PCR amplified, TA cloned and further subcloned into pGEX6P, pTrcHis and pcDNA3.1/His expression vectors. Following transformation into *E. coli*, positive transformants were identified by PCR using primer combinations complement to vector and *tat* sequences. Lanes 1 & 2, water and JDV genomic DNA, respectively, amplified with primers jTat4990 and jTat5328; lanes 3, 6 & 9, and 4, 7 & 10, blank and *tat* containing vectors, respectively, amplified using appropriate vector-specificprimers; lanes 5, 8 & 11*tat* vectors amplified with appropriate vector-specific forward primers in combination with jTat5328. Marker was 1 kb Plus DNA marker (InVitrogen).

Table 4.2. Expected size of amplicons following PCR screening of Tat expression constructs.

Primer set	Expected size (bp)					
	pGEX	pGEX/tat	pTrc/His	pTrc/tat	pcDNA/His	pcDNA/tat
Vector specific	189	526	245	582	260	597
Vector forward-insert reverse	–	414	–	483	–	493

Expression in *E. coli*

The *tat* gene exon 1 encoding 97 aa residues was expressed as a GST fusion protein with pGEX-6P and a His_6 fusion protein with pTrcHis vectors in *E. coli* following IPTG induction. Analysis of cell homogenates by SDS-PAGE showed a strong ~38 kDa and a weaker ~17 kDa band for GST- Tat and His-Tat fusion proteins, respectively (Figure 4.3A). The size of GST-Tat concurs with values estimated from the deduced amino acid sequences of 37.7 kDa (Table 4.3), while His-Tat migrated slower than the estimated 15 kDa. Their identity was confirmed by Western blotting analysis using anti-GST or anti-His_6 antibodies (Figure 4.3B). Densitometric analysis of the electrophoresed cell lysates suggested that GST-Tat was expressed at a higher level, about 17-20 % of total bacterial protein, compared to the His-Tat, which constituted about 5 % of total protein. Separation of IPTG-induced bacterial cultures into soluble and insoluble fractions clearly demonstrated that the recombinant proteins were insoluble, despite attempts to optimise conditions for the production of soluble protein. Several parameters were assessed including media for growth, temperature of growth, IPTG concentration, OD_{600} for induction and length of induction. The optimum yield was achieved following induction at OD_{600}= 0.5 with 0.1 and 0.5 mM IPTG for pGEX and pTrcHis, respectively, for 1.5 h at 37°C in either M9 or LB media.

Table 4.3. Predicted molecular weight and p*I* of recombinant and native Tat proteins. Calculation was done using ProtParam tool from ExPAsy.

Protein	Mw (kDa)	pI	No. aa
GST-Tat	37.7	8.8	330
His_6-Tat	15	9.7	135
Tabanan Tat	10.7	10.3	97
Pulukan Tat	10.7	10.2	97
Kalimantan Tat	10.7	10.5	97

When the induction was allowed for more than 2 h in the His-Tat system, the emergence of a lower sized protein band in the Western blots (Figure 4.4)

suggested protein degradation occurred, but degradation of GST-Tat did not appear to occur even when the induction continued for 4 h (data not shown).

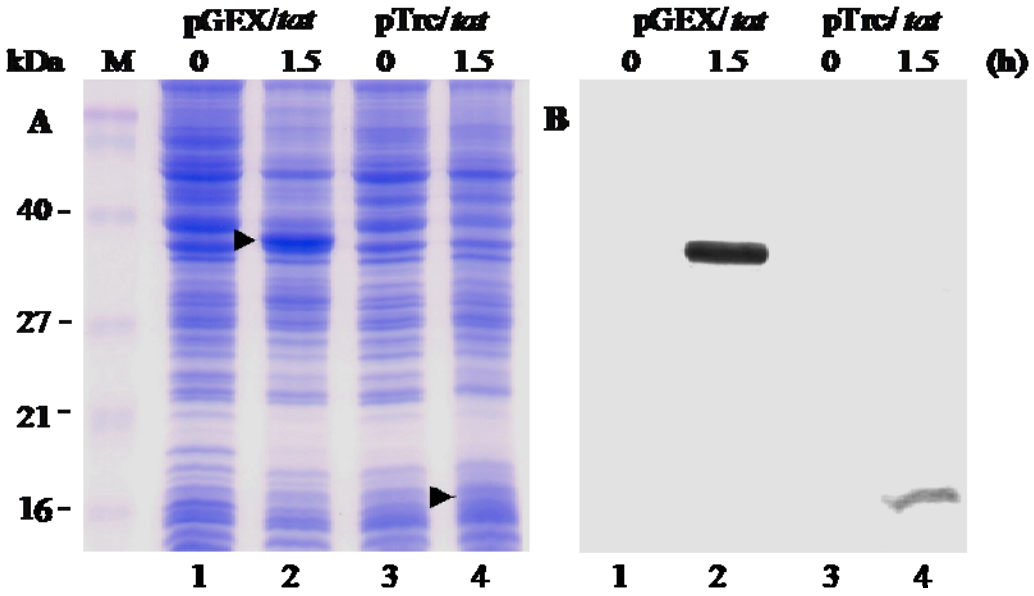

Figure 4.3. JDV Tat fusion proteins expressed in *E. coli.* (A) Total protein extracted from transformed Top10F' cells were fractionated on a 15 % polyacrylamide gel under reducing condition and visualised by Coomassie brillant blue staining. The bacteria were harvested prior (lanes 1 and 3) and after 1.5 h induction with IPTG (lanes 2 and 4). (B) Western-blot analysis using anti-GST and anti-His antibodies of the same preparation in A; higher level expression of 36-kDa GST-Tat (lane 2) was evident in comparison to the 17-kDa His-Tat (lane 4).

Purification of GST fusion protein

Only a small amount of soluble GST-Tat was recovered on the immobilised glutathione as demonstrated by Western bloting. As expected, the expressed protein, migrating at approximately 38 kDa, was detected using GST antibody, in addition to a smaller band, presumably unbound GST (lane 1, Fig 4.4). Greater yields were obtained by sequential detergent washing of the insoluble inclusion bodies (lane 3, Figure 4.4) followed by solubilisation using 6 M guanidium

chloride ((lane 4, Figure 4.4) or 8 M urea (lane 5, Figure 4.4). Several smaller GST-staining bands were detected in addition to the 38-kDa fusion protein, presumably indicative of protein degradation of the fusion protein, perhaps associated with harsh solubilisation conditions. There was no obvious difference in yield between these two denaturing agents. The protein concentration was described below.

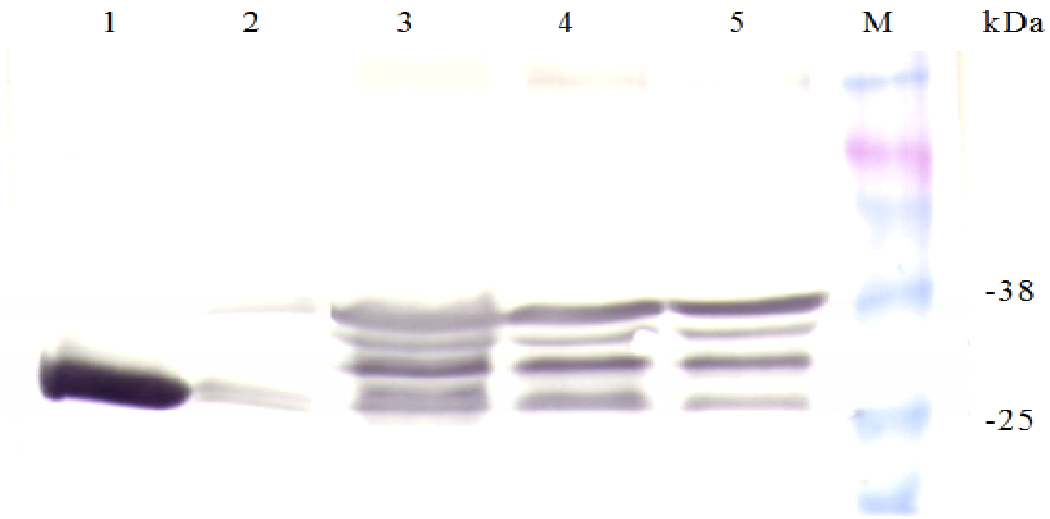

Figure 4.4. Western blot analysis of purified GST-Tat. Protein purification was initially done using Glutathione-Sepharose 4B (Sigma). Lane 1 and 2, GST control plasmid and GST-Tat plasmid, respectively, expressed in bacterial cells, demonstrating soluble GST or GST-fusion proteins. The insoluble protein was partially purified by detergent washing (lane 3) and further solubilised with 6M guanidium chloride (lane 3) or 8M urea (lane 4). The protein products were probed with anti-GST antibody to confirm identity and antigenicity.

Purification of His_6 fusion

The Ni-NTA resin (Qiagen) enabled the purification of His-labelled Tat in the inclusion body material solubilised with 6 M Gu-HCl or 8 M urea. Following recommended immobilisation, washing and elution protocols, the His-Tat protein of 17 kDa was detected from the eluates in considerable purity (Figure 4.5) in

comparison to the purified GST-Tat (Figure 4.7). Despite this apparent low impurity in CBB stained gel, several immunogenic bacterial protein contaminants produced high background reaction in WB assay using sheep Tat antiserum (data not shown).

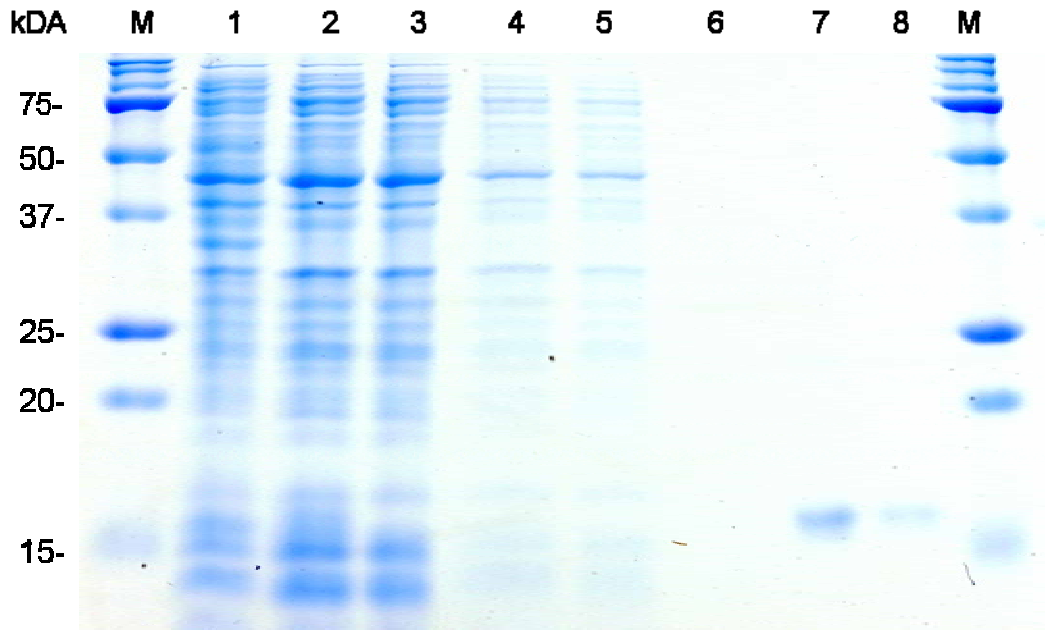

Figure 4.5. Purification of His-Tat. Following induction with 1 mM IPTG for 1.5 h at 37°C, the *E. coli* cells were lysed with buffer containing 8 M urea and centrifuged to pellet cell debris. The supernatant was applied to a Ni-NTA column and the His-Tat was eluted with buffer containing 250 mM imidazole. Lane 1,: solubilised non-induced cells; lane 2, solubilised IPTG-induced cells; lane 3, flow through; lanes 4–6, 1st, 2nd and 3rd washes; lanes 7–8, 1st and 2nd eluates.

Expression in mammalian cells

To reduce background contamination of the Tat proteins by *E. coli* proteins, His-Tat was expressed constitutively in mammalian cells using pcDNA/*tat* vector (His-Tat_{mam}). Transfected COS7 cells, which survived routine passage in media containing G-418 sulphate for 6 to 8 weeks, were subjected to protein analysis. Initial analysis on whole cell extracts was unable to detect the predicted protein, indicating low level expression in this system. After purification of the protein using Ni-NTA affinity chromatography, SDS-PAGE and subsequent silver staining

detected a major protein product of approximately 17 kDa in size (Figure 4.6, Panel A, Lane 2) that was bigger than the calculated molecular mass of 15 kDa as noted with the prokaryotic version mentioned earlier. The identity of the protein band migrating at 17 kDa was determined by Western blot using anti-His monoclonal antibody (Figure 4.6, Panel B, Lane 2) and sera from animals injected with GST-Tat (Figs. 4.9–11). Furthermore, the antigenic reactivity of this protein was confirmed by Western blot with bovine sera collected from endemic area (Table 4.4). JDV-negative bovine sera failed to recognize the protein, thereby showing the specificity of the assay.

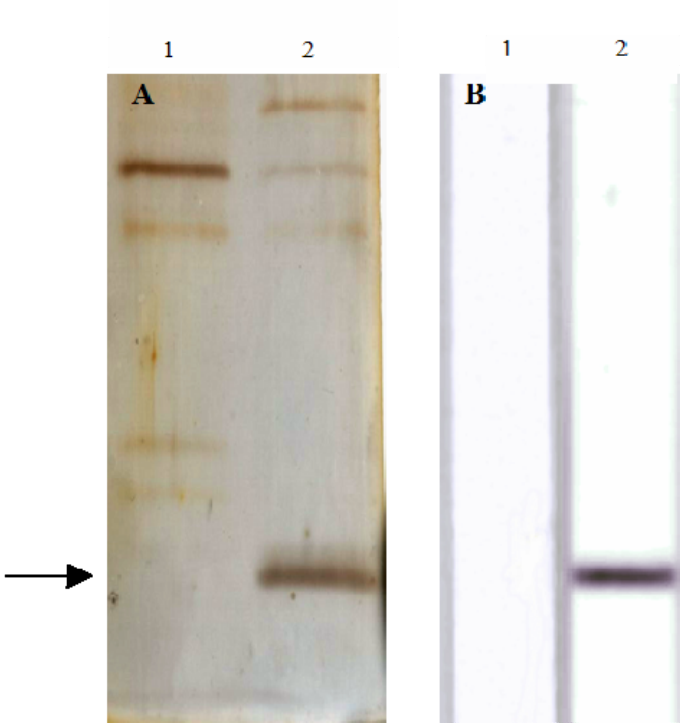

Figure 4.6. Expression and purification of His-Tat produced in COS7 cells stably transfected with pcDNA/*tat*. (A) The His_6-tagged Tat was purified using Ni-NTA affinity chromatography. Protein eluates from pcDNA3.1 (lane 1) and pcDNA/*tat* (lane 2) transfected cells were resolved in 15 % SDS-PAGE. Subsequent silver staining cells identified His-Tat_{mam} as a 17 kDa protein (arrow). (B) Western blot analysis identical to that shown in panel A was done using monoclonal anti-His which recognised the 17-kDa band.

Estimation of the protein concentration

As mentioned above, only a low yield of soluble GST-Tat was recovered on the immobilised glutathione after IPTG induction of the pGEX/*tat* transformed *E. coli* in contrast to the sequential detergent washing of the insoluble inclusion bodies followed by solubilisation using Gu-HCl or urea. Proportional to the known concentrations of BSA and lysozyme and the harvest volume, densitometric analysis calculated an estimated 3.5 mg of purified GST-Tat was produced from 200 mLculture with both denaturing agents compared to only 0.2 mg soluble protein (Lanes 6 and 7 Figure 4.7). While the yield of His-Tat produced in *E. coli* was estimated about 1 mg/200 mLculture (Lane 8 Figure 4.7), less than obtained with the GST-Tat system and approximately 80 µg His-Tat was purified from 4 x 75 cm^2 flasks of COS7 cells (Lane 9 Figure 4.7).

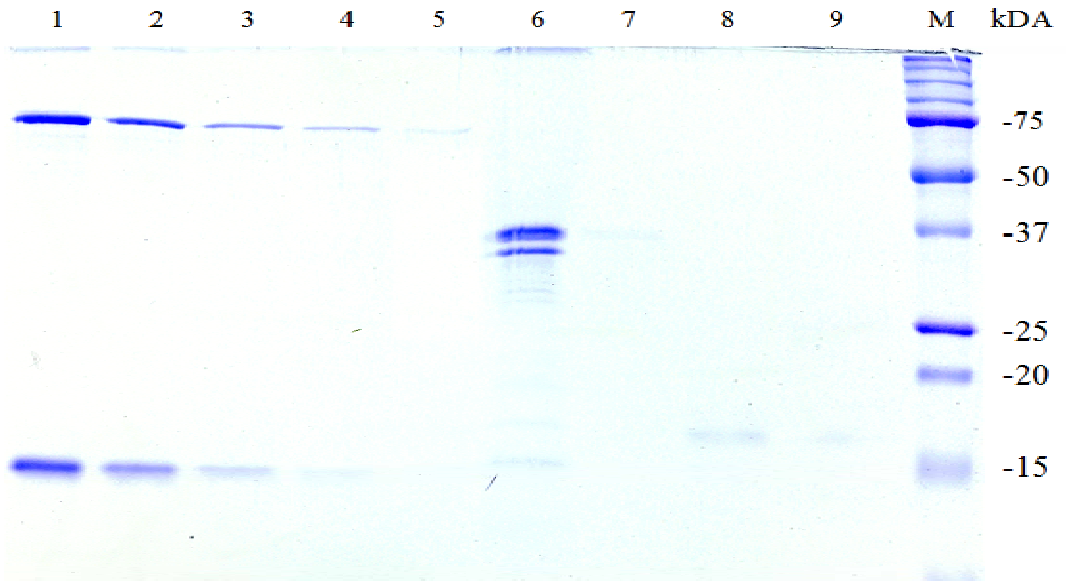

Figure 4.7. Concentration estimation of purified recombinant Tat proteins. Following SDS-PAGE of purified proteins and known concentrations of BSA (67 kDa) and lysozyme (14.3 kDa), concentration of Tat proteins was calculated densitometrically. Lanes 1-5, BSA and lysozyme in various concentrations of 1,000, 500, 250, 125 and 50 ng, respectively; lane 6, GST-Tat purified from insoluble and lane 7, soluble fractions; lane 8, His-Tat produced in *E. coli* and lane 9, His-Tat_{mam}.

Induction of Tat antibody in sheep and cattle

The immunogenicity of GST-Tat was confirmed by the induction of an antibody response following its injection in both sheep and cattle, detected by Western immunoblotting. In sheep, weak reactivity against the immunising antigen appeared to be present at the time of the initial inoculation and 2 weeks after the first injection, and increased in intensity following subsequent booster injections (Figure 4.8, Panel A). The weak signal given by pre-immune serum was presumably due to the presence of anti-GST antibody in the immunised animal. Since only partially purified GST-Tat antigen was used in this experiment, there was a high background due to contaminating *E. coli* proteins, which made the specificity of the antibody difficult to determine. Using an antigen prepared with the eukaryotic expression system (His_6-Tat in COS7 cells), which eliminated the background reaction associated with *E. coli*, the presence of Tat-specific antibody was evident (Figure 4.8, Panel B). The sheep antiserum was shown to have a titre of $\geq$400 (Figure 4.8, Panel B).

In 4 Bali cattle injected with a similar GST-Tat preparation, a strong specific antibody response was observed in 2 animals on day 28, 2 weeks after the second injection, and the intensity of the reaction increased at day 42 after an additional injection of the antigen (Figure 4.9). Weak reactions against Tat were detected in sera of the other two animals at 28 and 42 d (Figure 4.9); in these two animals there was also a weak response against the GST component of the immunogen, suggesting these 2 animals were poor responders. No reactivity to Tat was detected in animals immunised with GST only. These results showed that the eukaryotic recombinant Tat protein can be used as a test antigen for the serodetection in Tat vaccinated animals.

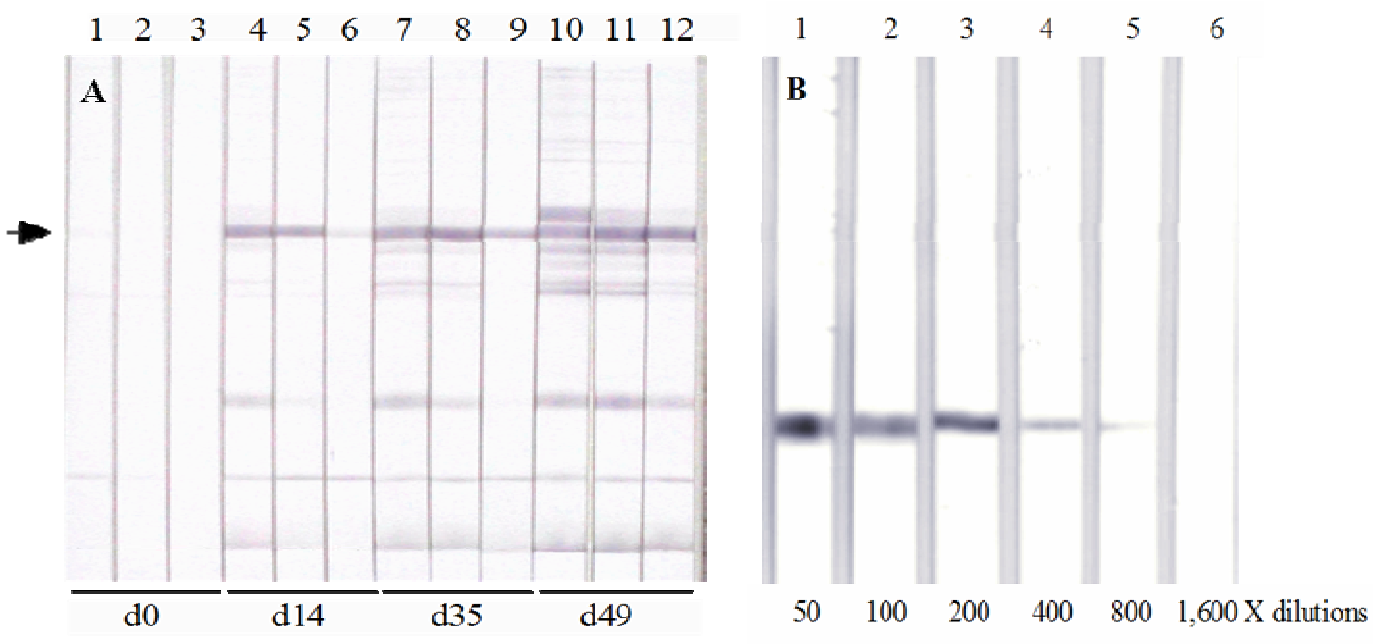

Figure 4.8. Detection of Tat-specific antibody following the injection of GST-Tat into sheep. (Panel A) The detergent washed GST-Tat used for immunisation was blotted and incubated with sheep antisera collected at different days (d) after injection as indicated. Lanes 1, 4, 7, 10; 3, 6, 12 and 2, 5, 8, 11, 1:100, 1:1,000 and 1:5,000 dilutions of serum, respectively. The arrow indicates the expected size of GST-Tat. (Panel B) Western immunoblot using a His-Tat_{mam} antigen produced in stably transfected mammalian (COS7) cells, demonstrating the titration of Tat-specific antibodies induced in sheep against a crude GST-Tat antigen.

The serum sample from animal CB46 collected at day 42 (Figure 4.9) was serially diluted and reactivity to Tat was moderate in comparison to GST (Fig 4.10), suggesting that Tat was a relatively weaker immunogen than GST.

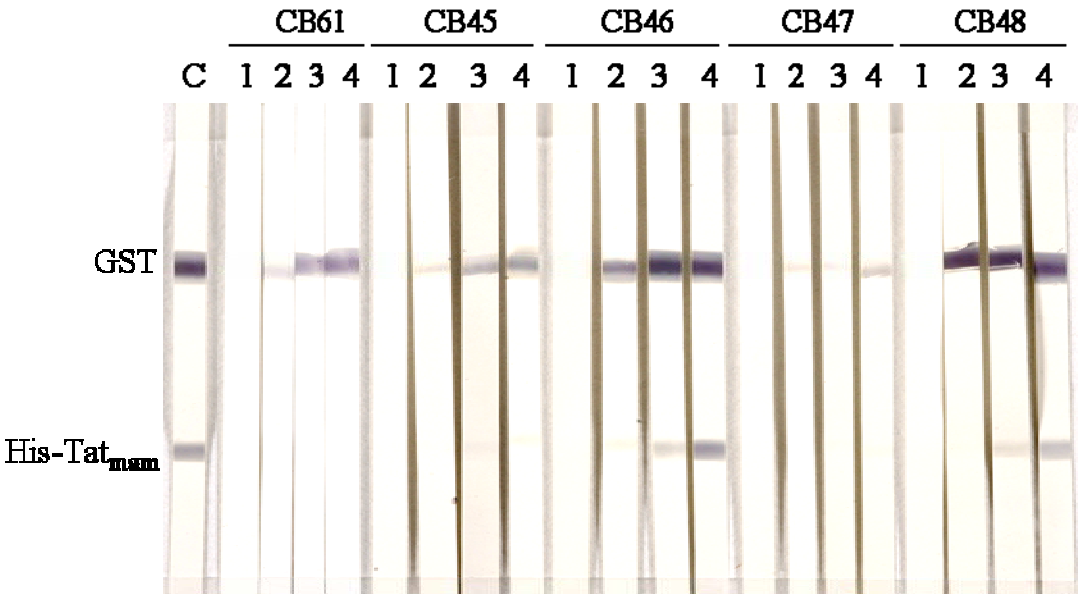

Figure 4.9. Western immunoblot demonstrating the antibody response in Bali cattle at days 0 (lane 1), 14 (lane 2), 28 (lane 3) and 42 (lane 4). Four animals were injected with a GST-Tat preparation at 0, 14 and 28 days;. Animal CB61 was injected with a control GST protein. Serum samples were diluted 1:20 and the blot incorporated both purified GST antigen and a His-Tat antigen produced in stably transfected mammalian cells (COS7). In 2 animals (CB46 and CB48), antibody against Tat was detected on day 28, 2 weeks after the second injection, and there was stronger reactivity 2 weeks after the third injection. In animals CB45 and CB47 there was weak reactivity against Tat at day 28 and 42, and no reactivity was detected against Tat in animal CB61 injected with GST only. Lane C was reacted with sheep anti-GST-Tat serum.

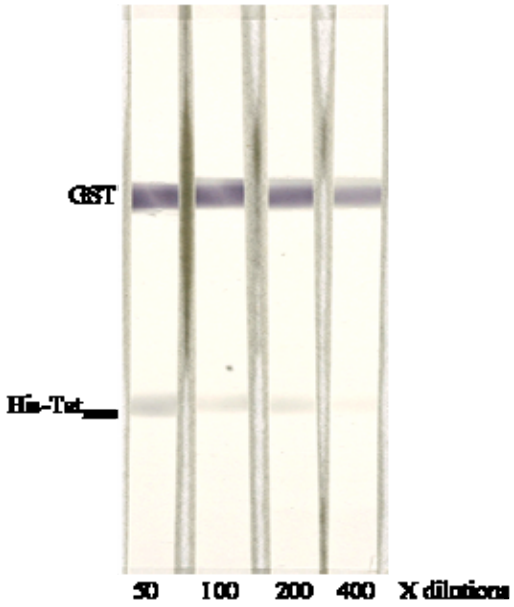

Figure 4.10. Titration of antibodies from animal CB46 shown in Figure 4.9. Serum samples from animal CB46 were further diluted as indicated and reacted against GST and His-Tat$_{mam}$. A weak reaction to Tat was shown at a 1:400 dilution of the serum in comparison to the strong reaction to GST.

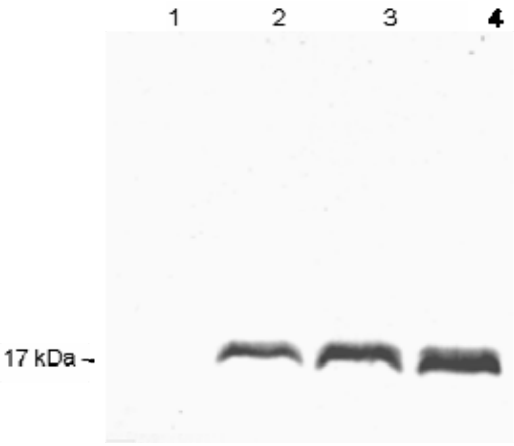

Figure 4.11. Western blotting demonstrating His-Tat expression after varying induction times with 1 mM IPTG. Cell lysates were prepared before (lane 1), 1 h (lane 2), 1.5 h (lane3) and 2 h (lane 4) after induction. The proteins of 17 kDa were detected with an affinity purified sheep anti-Tat antibody.

To eliminate non-Tat antibodies in the polyclonal sheep anti-Tat, the serum was affinity purified by adsorption of antibody onto GST-Tat and then by removal of non-specific antibody by absorption against GST and bacterial proteins. This affinity-purified serum recognised only a single band of approximately 17 kDa when reacted with the whole cell lysate of His-Tat expressing *E. coli* as shown in Figure 4.11, which confirms its specificity.

Detection of JDV Tat native protein

PMBC and spleen lysates collected from Bali cattle during experimental infection with each of 3 different JDV strains were used as the protein sources. Western blotting using affinity purified monospecific sheep anti-Tat detected protein bands with apparent mobility of 14 kDa primarily from PBMCs obtained prior to febrile period (Figure 4.12, panel A). The seroreactivity of the 14 kDa protein was comparable with the eukaryotic His-Tat_{mam} (Figure 4.12, panel B). As noted with the His-Tat proteins, the native Tat proteins produced by Tabanan/87, Pulukan and Kalimantan JDV strains also migrated slower than 10.7 kDa predicted from the DNA sequences specifying 97 aa (Table 4.3). This apparent mobility anomaly was also observed with Tat protein of BIV and HIV (Fong *et al.*, 1997; Goh *et al.*, 1986).

Seroreactivity of the Tat protein in different population of Bali cattle

To further demonstrate the immunogenicity of JDV Tat, 147 serum samples were tested at a 1:20 dilution for the presence of anti-JDV antibodies by the recombinant protein-based Western blot using eukaryotic recombinant JDV Tat and GST-CA as test antigens. Tat antibody was detected in animals that had recovered from experimental infection (Table 4.4). As shown in Figure 4.13; however, the sera reacted weakly against Tat in contrast to the strong reactivity to capsid. Apparent reaction was mainly demonstrated by sera collected 1-month pi which was evident in two animals (lanes A1 and B1 Figure 4.13) and weakened by the time, while in animal C the response was dubious (lane C2).

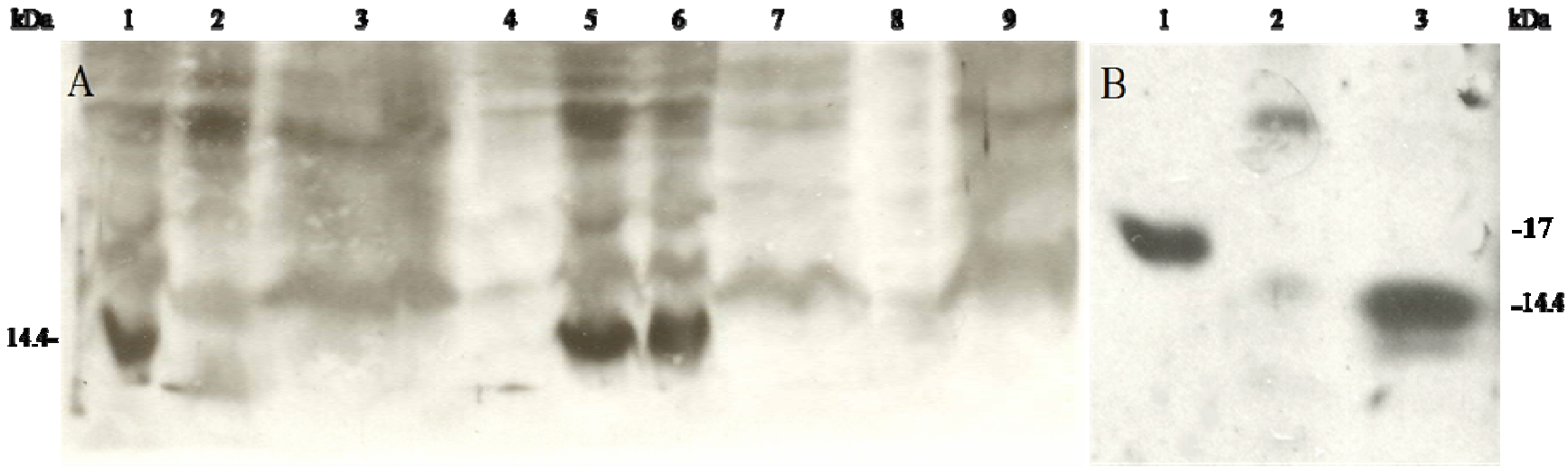

Figure 4.12. Detection of native JDV Tat protein in JDV-infected cattle. (A). Affinity purified sheep anti-Tat recognised a band of approximately 14 kDa in PBMC lysates collected immediately preceding the development of fever in cattle infected with Tabanan/87 (lane1), Pulukan (lane 5) and Kalimantan (lane 6) strains of JDV. No reactivity was shown by spleen (lanes 2, 4 and 8) and PBMC lysates (lanes 3, 7 and 9 representing Tabanan, Pulukan and Kalimantan strains, respectively) collected on the second day of the febrile reaction. Immunoblots were developed using ECL detection system (Amersham). (B). High resolution 16 % Tricine-gel was used to for better protein separation, and the eukaryotic His-Tat_{mam} was included (lane 1) as a comparison. The native Tat (lane 3) migrated relatively faster (14.4 kDa) than the His-Tat (17 kDa); both of them migrated slower than the predicted molecular weight of 15 and 10.7 kDa, respectively. Lane 2 is PBMC lysate from an animal recovered from the acute phase disease as in panel A.

Table 4.4. Prevalence of Tat antibodies in Bali cattle and correlation with ELISA and presence of antibodies to the JDV capsid protein.

JDV Status	No. sera tested	Absorbance reading of ELISA[a]	No. (%) WB positive[b]	
			Capsid	Tat
Recovered cattle	3	NA	3 (100)	2 (75)
Vaccinated cattle[c] (Bali and Kalimantan)	54	> 3.0	54 (100)	27 (50)
	61	1.1–3.0	61 (100)	1 (1.6)
Unvaccinated cattle (Bali)	10	ND	10 (100)	2 (20)
Jembrana disease-free area (South Sulawesi)	10	ND	1 (10)	1 (10)
Jembrana disease-free area (Nusa Penida)	5	Neg	0 (0)	0 (0)

[a]ELISA was done by Disease Investigation Centre, Bali utilising whole virus pelleted from plasma as an antigen (Hartaningsih *et al.*, 1994).

[b]Antibody detected by Western blotting using a recombinant JDV capsid (Burkala *et al.*, 1998) and recombinant Tat blotted together in the same strip as shown in Figures 4.14 and 15.

[c]Cattle were vaccinated with a crude spleen vaccine (Hartaningsih *et al.*, 2001)

NA and ND denote not available and no done, respectively.

Antibody was detected in 24 % of 115 vaccinated cattle in Bali and South Kalimantan; these had all tested positive by an ELISA utilising a whole virus antigen and were positive for antibody to recombinant capsid protein by Western immunoblots (Table 4.4). In general, the seroreaction signal to Tat was weaker relative to capsid (Figure 4.14); relatively strong positive Tat results usually correlated with high ELISA titres (Table 4.4) and strong recognition to capsid antigen (lanes 1–5, Figure 4.14). While sera with lower ELISA titre was less reactive to capsid and did not show reactivity with Tat (lanes 14–16, Figure 4.14). In cattle without vaccination history, Tat antibody was detected in 2 of 10 sera (Table 4.2) that were positive for antibody to the JDV capsid protein and were presumed to have been infected previously with JDV.

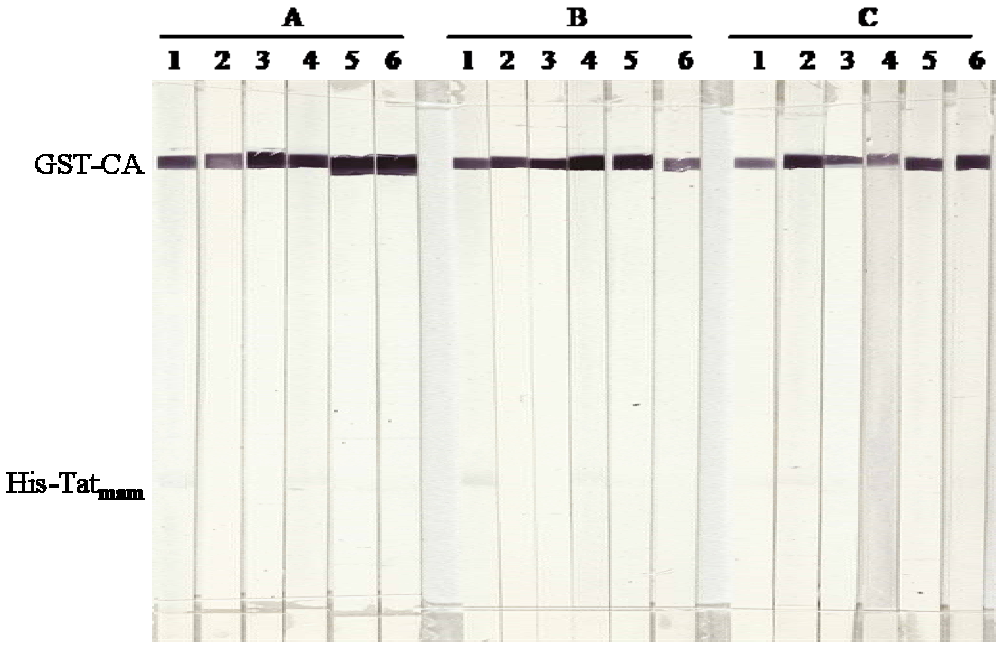

Figure 4.13. Reactivity of bovine sera against capsid (CA) and Tat proteins in experimentally infected cattle (A-C). Convalescent sera were collected monthly for 6 months (1-6) post infection. Weak reactivity to Tat was shown by sera collected 1-month post infection and reactivity decreased further with time, in contrast to the reactivity to CA. Sera were tested at a 1:20 dilution.

Tat antibody was also detected in one serum from South Sulawesi where Jembrana disease has not been recognised; the serum was also reactive against capsid protein (Table 4.4). Whereas Tat antibody was not detected in Bali cattle from the Jembrana disease-free island Nusa Penida (Table 4.2); these cattle were also negative for antibody to JDV capsid protein as represented in lane 16, Figure 4.14.

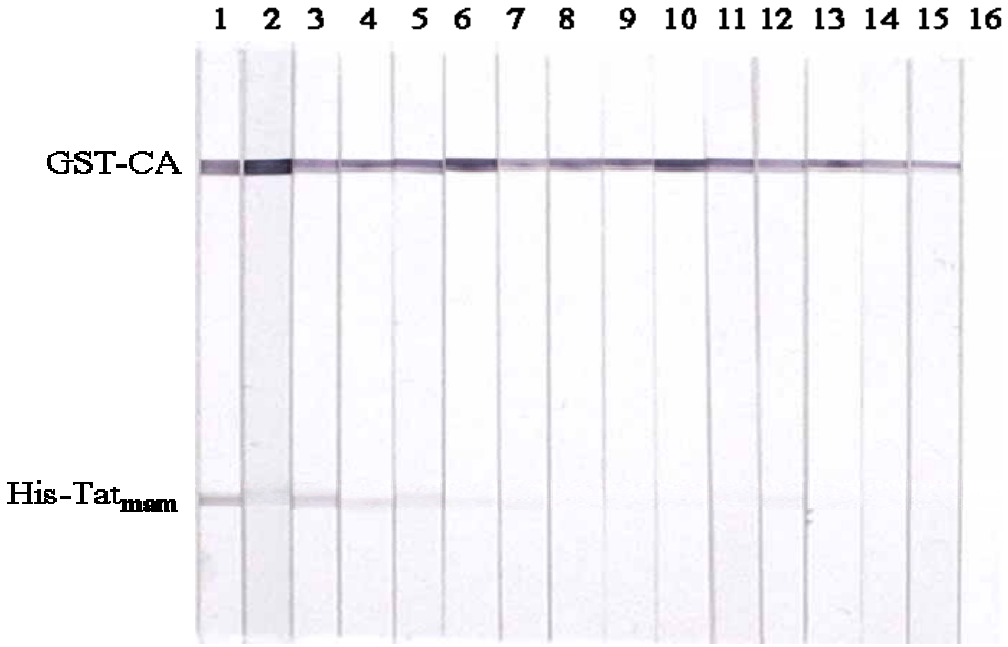

Figure 4.14. Reactivity of bovine sera against CA and Tat in vaccinated cattle. Immunoreactivity was mostly demonstrated by cattle vaccinated with inactivated tissue-derived virus antigens (lanes 1-15) as shown in Table 4.2. Tat reactivity was shown mostly by animals with high ELISA titre (Table 4.4) and strong reactivity to the JDV capsid protein. Lane 16, serum sample from disease-free area of Nusa Penida (Table 4.4). Sera were tested at 1:20 dilution.

Discussion

The methods selected for the production of recombinant proteins were partly based on their intended use. There were three basic needs: first, a method for production of potential antigens that could be used in vaccines, that would provide a high level of protein expression and would be appropriate for use in Indonesia; second, potential vaccine proteins would need to be immunogenic and preferably soluble; third, proteins intended as antigens for serological tests would need to have minimal or no antigenic cross-reactivity with non-JDV proteins, so providing high specificity. For the production of a JDV Tat antigen that might be incorporated into a vaccine and could be used in Indonesia, bacterial expression system were considered preferable because of relative simplicity of production

methods and potential yields, thereby reducing costs. Either a GST or His-tagged protein seemed appropriate for a vaccine. For a serological antigen, where high level production was less important, two systems were considered appropriate: either a His-tagged (fusion) protein expressed in a bacterial system, or one that would enable expression in eukaryotic cells. His-tags enable single-step affinity purification using nickel-chelating resin under denaturing condition, and the eukaryotic (COS7 cell) system eliminates the problem of cross-reactivity with contaminating *E. coli* proteins.

High levels of expression were achieved with the pGEX system producing a GST-Tat fusion protein. The GST fusion partner is well known to increase the quantity and stability of expressed proteins (Hearn & Acosta, 2001). This system was considered a potentially efficient way of producing JDV Tat antigen suitable for incorporation into a vaccine. However, although yields were high in this prokaryotic system, the yield of soluble protein was low, too low for efficient large-scale production. The proteins were produced mainly as insoluble inclusion bodies. Formation of inclusion bodies is known to be a common outcome in over-expression of heterologous protein in *E. coli* cytoplasm (Hannig & Makrides, 1998). Findings similar to these have been reported with HIV-1 Tat expressed with different vectors in bacterial systems: the high cysteine content of Tat has been implicated in this aggregation as it causes misfolding of the protein through intra- and/or inter-molecular binding (Frankel *et al.*, 1988a; Frankel *et al.*, 1988b; Kirsch *et al.*, 1996; Koken *et al.*, 1994; Rhim *et al.*, 1994). Nine cysteine residues were predicted to occur in JDV Tat1 (Chadwick *et al.*, 1995b). Partial purification of inclusion bodies was eventually achieved following solubilisation with either Gu-HCl or urea, even though re-aggregation was evident after dialysis against PBS. These procedures did not appear to affect the antigenicity and immunogenicity of the protein, and refolding was not attempted. Urea provides a potentially low cost method for the large scale solubilisation of protein for preparation of a vaccine, should such a vaccine be shown efficacious against JDV.

This aggregation propensity was anticipated using a His_6 tag expression system, pTrc/His. A strong and conformationally independent interaction between 6 histidine tags and immobilized Ni^{2+} ions has been the main advantage of this system, which allows protein purification under denaturing conditions (Hochuli *et al.*, 1987; Holzinger *et al.*, 1996). Thus, proteins that have aggregated into inclusion bodies can be dissolved in a suitable agent such as urea before affinity purification. Unfortunately, despite the relatively higher purity the yield obtained using this system was less than the GST system. However, for future large scale production, further scale-up optimisation of this system is worth considering.

To enable a confident assessment of antibody response induced by the bacterially expressed recombinant Tat, an unrelated expression system was employed to synthesise the JDV Tat in pcDNA3.1/His vector. This vector contains the cytomegalovirus immediate early promoter (P_{CMV}), which provides a high level constitutive expression in a range of mammalian cells (Boshart *et al.*, 1985), and a bovine growth hormone polyadenilation (BGHpA) signal for efficient transcription termination and polyadenylation of mRNA (Goodwin & Rottman, 1992). The advantages of mammalian cells over other expression systems are the ability to carry out proper protein folding, complex glycosylation, as well as a broad spectrum of post-translational modifications (Geisse *et al.*, 1996). As expected, the His-Tat protein expressed by this system (His-Tat_{mam}) reacted specifically with serum antibodies collected from GST-Tat injected animals. The clean background of the reaction may provide a diagnostic reagent for analysing antibody response to Tat vaccination unambiguously.

The immunogenicity of the GST-Tat fusion protein was initially established by the ability of this preparation to induce specific antibody response in sheep, and subsequently in Bali cattle in Indonesia. Comparable immunogenicity was observed in immunised animals, although a relatively greater immunological response to the GST rather than the Tat moiety was noted (Figures 4.8-4.10), perhaps due to the insoluble nature of the immunizing antigen (Rothel *et al.*, 1997) or simply because JDV Tat was relatively less immunogenic than the GST. In all 4 cattle immunised with GST-Tat, antibody was detected (Figure 4.9)

although the level of antibody seemed to vary markedly between the animals. This could be significant if the GST-Tat was trialled as a vaccine in Bali cattle. However, the level of Tat antibody induced using this GST-Tat preparation was higher than using the conventional vaccine. The result, therefore, indicates that the insolubility of the immunising GST-Tat did not affect its final immunogenicity.

The antigenicity of GST-Tat was confirmed by the demonstration that the sheep anti-Tat antibodies were reactive with native JDV Tat produced in cattle infected with each of Tabanan/87, Pulukan and Kalimantan strains which indicates the conservation of the immunogenic/antigenic domain in support to genetic studies in Chapter 3. The native JDV Tat was shown to have an apparent molecular weight of approximately 14 kDa, which was greater than that (10.7 kDa) calculated from the deduced 97 aa sequence (Table 4.3). Similar findings to this were described for BIV and HIV-1 Tat proteins, both were identified as 14 kDa as opposed to the predicted 12.7 and 10.7 kDa, respectively. These discrepancies thought to be associated with high pI or number of positively charged (basic) residues such as arginine and lysine (Aldovini *et al.*, 1986; Ensoli *et al.*, 1993; Fong *et al.*, 1997; Goh *et al.*, 1986; Jeang *et al.*, 1988; Wright *et al.*, 1986). Protein with high basic residues may be analysed to have an apparent molecular weight greater than the actual molecular weight because basic amino acids attract more negatively charged SDS, resulting in the increase of the mass to charge ratio of the SDS-protein complex, thus lowering electrophoretic mobility (Hames, 1998). This effect was also observed in His-Tat proteins, but not in GST-Tat probably due to the greater mass and lower pI of the latter protein (Table 4.3).

The prevalence of Tat antibody in seropositive cattle was low (Table 4.4). The weaker signal to Tat protein observed in this study indicates the low titre of naturally occurring Tat antibody as a result of infection or immunisation with the crude tissue vaccine. Tat antibody was only weakly detected in convalescent sera from experimentally infected cattle despite the strong reactivity to JDV capsid protein in these animals, and 2 out of 10 sera from unvaccinated cattle in endemic areas were reactive; presumably these cattle had recovered from natural

infection. One serum sample from the Jembrana disease free area of South Sulawesi reacted with Tat and CA proteins suggesting that an antigenically-related non-pathogenic bovine lentivirus is circulating in this area. Results from experimental infection suggest that antibody response to Tat is transient in contrast to CA. This discrepancy might be due to the different abundance of the 2 proteins during the course of infection and possibly the immunological status of the infected animals. These results suggested that Tat protein was expressed early in the infection and appeared to be a minor protein similar to what was previously reported in HIV-1 infection (Chang *et al.*, 1997; Ensoli *et al.*, 1993). Possibly, therefore, only small amount of JDV Tat was presented to the humoral immune system during viral infection or in vaccination with inactivated vaccine. This conclusion; however, needs to be confirmed.

Like the pathogenesis of disease associated with many lentivirus infections, in both man and animals (Haffer *et al.*, 1990; Newman *et al.*, 1991; Zhang *et al.*, 1997), the immune system in JDV-infected cattle is severely damaged; there is a marked lymphopenia (Soesanto *et al.*, 1990) and generalised atrophy of follicular (B cell) areas of lymphoid tissues (Dharma *et al.*, 1991) which would account for the delayed immunological response. The antibody response to JDV in infected animals is delayed (Hartaningsih *et al.*, 1994; Wareing *et al.*, 1999), usually not occurring for several weeks after infection and recovery.

Despite evidence suggesting a strong correlation between anti-Tat antibody and delayed AIDS progression (Agwale *et al.*, 2002; Barillari *et al.*, 1999; Pauza *et al.*, 2000; Reiss *et al.*, 1991), it remains to be confirmed whether the development of Tat antibody in cattle is associated with protection against infection. There is no clear evidence from the serological results of vaccinated and recovered cattle that this is true. Cattle recovered from JDV infection have been shown to resist further challenge with virulent virus (Soeharsono *et al.*, 1990) but Tat antibody appeared to be transient and was detected in only some cattle that were recovered from experimental or natural infection and were all positive for antibody to the immunodominant capsid protein. Tat was certainly less immunogenic than the capsid protein but possibly, low levels of Tat antibody were present, beyond

the limit of detection, but sufficient to provide a protective immunity. The use of epitope peptide in ELISA test would be expected to increase sensitivity and specificity of serological detection in clarifying this question. The low seroprevalence and antigenicity of JDV Tat was not unexpected; similar findings in HIV-1 have been reported (Butto *et al.*, 2003; Krone *et al.*, 1988).

In conclusion, given that Tat antibody has inhibited HIV 1 replication *in vitro*, and lowered viral load and delayed disease progression in seropositive patients (Mhashilkar *et al.*, 1995; Re *et al.*, 1995; 2001b; Reiss *et al.*, 1990; Zagury *et al.*, 1998b), it seems worthwhile to investigate the role of Tat antibody in the development of protective immunity against JDV infection. The studies reported in this Chapter have paved the way for such studies. It was clearly demonstrated that recombinant Tat proteins could be produced that were immunogenic in cattle. The evaluation of Tat vaccines in the induction of a protective immunity seems a logical extension of these studies. Application of different adjuvants and vaccination dosage are warranted to modulate the immune responses for a better outcome. Such studies are currently under investigation in Indonesia.

Chapter 5

Construction and *in vitro* evaluation of a DNA plasmid encoding JDV *tat*

Summary

Preliminary studies towards the development of a potential JDV *tat* DNA vaccine are described. A plasmid encoding the Tat protein was constructed based on the mammalian expression vector pVAX1. The performance of this construct was evaluated via transfection into COS7 and primary bovine lung cells. Transcription of the encoded gene was confirmed through the detection of *tat* mRNA by RT-PCR. The presence of Tat protein in the transfected cells was detected by immunofluorescence staining; the protein was shown to localise primarily in the nuclear pheriphery. Further verification of the functional performance of this construct was established using a *trans*-activation assay. The results demonstrate that the *tat* DNA plasmid is functional *in vitro* and further evaluation in animals is warranted.

Introduction

In Bali cattle, JDV infection results in a transient high titre of infectious virus (virus load) coincident with the febrile period and acute disease syndrome. The virus load declines after the fever has dissipated (Soeharsono *et al.*, 1990). An antibody response is not detected for several weeks after infection (Hartaningsih *et al.*, 1994), presumably associated with lesions in the lymphoid system and particularly with depletion of follicular (B cell) areas (Dharma *et al.*, 1994). The delayed antibody response suggests the potential importance of cell-mediated immunity (CMI) in recovery from infection and perhaps in the suppression of JDV infection in naïve animals. There is evidence that strongly suggests the essential role of this arm of the immune responses in containing lentivirus infections including HIV-1 (Borrow *et al.*, 1994; Koup *et al.*, 1994), SIV (Kuroda *et al.*, 1999; Schmitz *et al.*, 1999), EIA (Hammond *et al.*, 1997; McGuire *et al.*, 1994; 1997) and FIV (Flynn *et al.*, 1996; 2000). In addition, there is evidence that cytotoxic T lymphocyte (CTL) response against Tat may be more efficacious in controlling infection than comparable responses to viral structural proteins (Addo *et al.*, 2001; Allen *et al.*, 2000; Stittelaar *et al.*, 2002; van Baalen *et al.*, 1997). Relevant to these notions, there has been increasing interest in AIDS vaccine approaches that elicit CTLs, which recognize and eliminate cells infected with HIV (McMichael & Hanke, 2003). Unlike antibodies, effective CTL responses can be directed against epitopes derived from any viral protein, raising the possibility that CTLs can be targeted to regions that are more conserved than the viral envelope.

A novel vaccination strategy involving the direct inoculation of a plasmid DNA containing appropriate genes encoding protein antigens involved in the induction of a protective immunity has been demonstrated to be an effective method of vaccination (Tang *et al.*, 1992). This DNA enables endogenous antigen processing mimicking that which might occur in natural infections and leading to the induction of antibody and a cell-mediated immune response (An & Whitton, 1999; Arulkanthan *et al.*, 1999; Bagarazzi *et al.*, 1998; Deng *et al.*, 2000). Protection as a consequence of the inoculation of the appropriate DNA has been

observed in many different infectious disease models (Ulmer *et al.*, 1993; Wang *et al.*, 1993), in native animals such as dogs and cats (Cherpillod *et al.*, 2000; Jallet *et al.*, 1999; Kwang *et al.*, 1999), pigs (Gerdts *et al.*, 1997; Hong *et al.*, 2002; Markowska-Daniel *et al.*, 2001), horses (Cantlon *et al.*, 2000; Lunn *et al.*, 1999) and cattle (Braun *et al.*, 1998; Harpin *et al.*, 1999; Loehr *et al.*, 2001; Schrijver *et al.*, 1997).

Tat has been used with success as a DNA vaccine component for the suppression of HIV infection in mice and cynomolgus monkeys (Billaut-Mulot *et al.*, 2001; Cafaro *et al.*, 2001; Caselli *et al.*, 1999) and CAEV infection (Beyer *et al.*, 2001; Harmache *et al.*, 1998). In this Chapter a JDV Tat-encoding plasmid was constructed and its function was evaluated in mammalian cell cultures.

Materials and methods

Vectors and cells

pVAX1 (Invitrogen) shown schematically in Figure 5.1, was used to drive expression of the inserted gene from the CMV immediate early promoter in mammalian cells. pVAX1/*lac*Z (Invitrogen) as shown in Figure 5.1, expresses the bacterial β-galactosidase gene and was used to determine the transfection efficiency and to generate a reporter plasmid for *trans*-activation assays.

The expression of an inserted gene in pVAX1 is driven by the CMV immediate early promoter (Boshart *et al.*, 1985; Nelson *et al.*, 1987) and terminated by BGH pA signal for efficient polyadenylation of mRNA (Goodwin & Rottman, 1992).

Plasmid constructs

Construction of pVAX1/tat

The first coding exon of JDV *tat* was synthesized by PCR using the forward primer jtatV1 5'-ACCGGCTAGCCaAATATGgCTG-3' (JDV nt.4994–5016) and the reverse primer jtatV2 5'-TTCCAGGGTCCAACGATCTAGTG-3' (nt.5306–5329)

from spleen DNA (Chapter 3). An *Nhe*I restriction endonuclease cleavage site (underlined) outside the coding sequence was included to facilitate further cloning. Two mutations (lowercase) were also introduced into the forward primer to form a strong ribosome-binding site AnnATGG (Kozak, 1997).

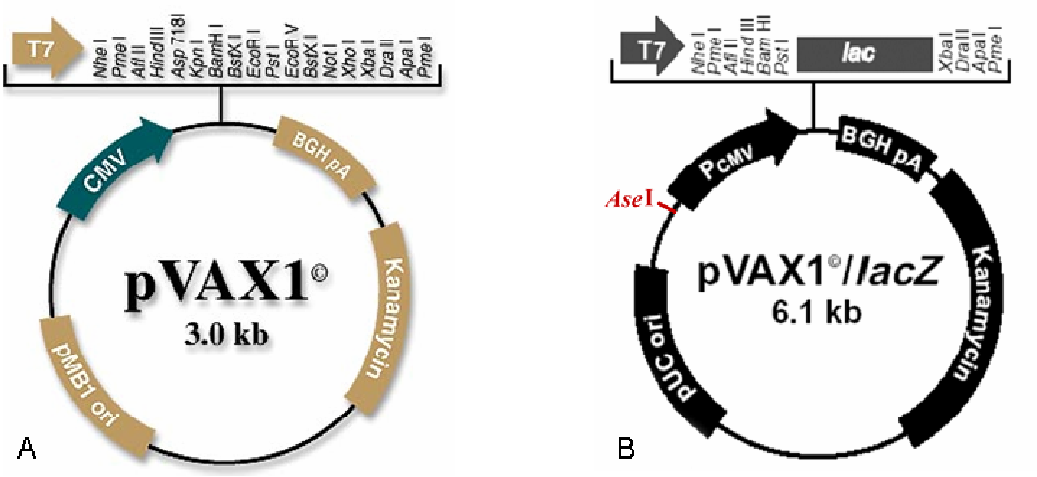

Figure 5.1. Schematic representation of mammalian expression vectors pVAX1 and pVAX1/*lac*Z. (A) pVax1 was used to generate a DNA vaccine by inserting JDV *tat* into *Nhe*I/*Eco*RI restriction sites. (B) pVAX1 encoding the 3.1 kb *lac*Z was used in transfection efficiency determination and reporter plasmid construction. To construct a reporter plasmid pLTR/*lac*Z, the CMV promoter was excised by *Ase*I/*Nhe*I restriction digestion and replaced by the JDV LTR promoter fragment.

The PCR reaction was done using Expand™ high fidelity PCR system (Roche) and was prepared as detailed in Chapter 3. After initial incubation at 95°C for 5 min, the reaction was allowed to proceed for 30 cycles (95° for 40 s, 69°C for 30 s and 72°C for 1 min), followed by a final incubation at 72°C for 7 min. The amplified product was gel purified,TA-cloned and subsequently sequenced. To generate a DNA vaccine that expresses the JDV Tat, pVAX/*tat*, the *tat* fragment was cleaved from the TA-vector with *Nhe*I and *Eco*RI restriction endonucleases and fused to the respective sites of pVAX1 vector downstream of the CMV promoter (Figure 5.1A). Positive constructs were initially screened by PCR using

primers T7 in combination with BGH or jtatV1 (Table 5.1) followed by restriction enzyme digestion and subsequently confirmed by sequencing.

Table 5.1 Oligonucleotide primers specific to pVax1 DNA vaccine vector or JDV regions.

Primer	Sequence (5' to 3')	Genomic positions	
		Nucleotide	Region
T7	TAATACGACTCACTATAGGG	664–683	pVAX1
BGH	TAGAAGGCACAGTCGAGG	823–840	pVAX1
VAX	ACTCTTCGCGATGTACGG	2–19	pVAX1
jLTR1	AGCaTTaatCGGAGCTGGAATATCTGATTG	7424–7453	JDV
jLTR2	GTGCCTTACAGGCTAgCAGCTGGG	7678–7655	JDV
jtatV1	ACCGGCTAGCCaAATATGgCTG	4994–5016	JDV
jtatV2	TTCCAGGGTCCAACGATCTAGTG	5329–5306	JDV

Lowercase letters indicate nt modification introduced to create restriction enzyme cleavage sites (underlined) and ribosome-binding site

Construction of a reporter plasmid pLTR/lacZ

The JDV LTR containing basal promoter, enhancer elements and TAR region excluding the inhibitory element reported earlier (Chen *et al.*, 1999) was isolated by PCR from Tabanan/87 infected spleen DNA. Primers jLTR1 5'-AGCaTTaatCGGAGCTGGAATATCTGATTG-3' and jLTR2 5'-GTGCCTTACAGGCTAgCAGCTGGG-3' modified (lowercase) to contain *Ase*I and *Nhe*I sites (underlined) respectively were used to generate a 255 bp (nt 7424–7678) fragment with the same conditions as for *tat* except an annealing temperature of 65°C was used. The product was then TA-cloned and sequenced.

Following confirmation by sequencing, *Ase*I and *Nhe*I restriction enzymes were used to excise a 639-bp fragment containing the CMV promoter from pVAX1/*lacZ* (Figure 5.1B). The LTR promoter of 243 bp in size was cleaved with the same enzymes and fused to replace the P_{CMV} to generate pLTR/*lacZ*. An oligonucleotide VAX 5'-ACTCTTCGCGATGTACGG-3' corresponding to the pVAX1/*lacZ* region upstream of the *Ase*I site was used, in conjunction with the

BGH primer, for PCR screening using the conditions described in Chapter 3 except that the extension step was at 72°C for 3 min due to the larger size of the product and for sequencing.

Isolation of plasmid DNA

To obtain sufficient amounts of plasmid DNA for transfection experiments, the plasmid was purified using a Quantum Prep™ Midiprep (BioRad) according to the manufacturer's instructions. First, each plasmid construct (described above) was transformed into *E. coli* Top10 as detailed in Chapter 3. A single colony was selected and inoculated into 50 mLLB broth and incubated overnight at 37°C with vigorous shaking. The cells were pelleted by centrifugation (4,000 *g*, 10 min) and resuspended in 5 mLCell Resuspension Solution. Five mLof Cell Lysis Solution was added to lyse the cells, and then an equal amount of Neutralisation Solution was added. The mixture was centrifuged (8,000 *g*, 30 min) and the supernatant transferred into a new tube and mixed with 1 mLof matrix. After centrifugation (8,000 *g*, 2 min), the matrix was washed with 10 mLWash Buffer, centrifuged and resuspended in 600 µl Wash Buffer, subsequently transferred into a spin column. The column was centrifuged (14,000 *g*, 30 seconds) and washed once more with 500 µl Wash Buffer, followed by centrifugation (14,000 *g*, 2 min) to remove residual ethanol. Finally, 600 µl of warm deionised H_2O was added to the column, it was centrifuged (14,000 *g*, 2 min) to elute the DNA stored at –20°C. The DNA concentration was determined as previously described (Chapter 3).

Transient transfection

The ability of pVAX/*tat* to express Tat *in vitro* was determined in primary foetal bovine lung (FBL) cells and African COS7 cells. Both cell types were maintained and passaged as described previously (Chapter 4).

The detailed protocol for transfection was as previously described for pcDNA3.1/*tat* (Chapter 4) except that G-418 sulphate step for selection of transfected cells was omitted. Briefly, cells were seeded into either 6-well plates containing glass coverslips or 25 cm^2 flasks the day before lipid-mediated

transfection (Felgner *et al.*, 1987) was attempted. Transfection mixtures were prepared containing 5 µg pVAX1/*lac*Z, or 5 µg pVAX1, or 4 µg pVAX/*tat* plasmid DNA in serum-free DMEM with an additional 25 µl Lipofectamine^{TM} 2000 reagent (Invitrogen) per ml. Volumes of 200 µL or 1 mLwere applied to approximately 80 % confluent monolayers per well or per 25 cm^2 flask, respectively, and incubated at 37°C for 3h. The transfection medium was replaced with growth medium and the cells were incubated for an additional 48 h. Transfection experiments were performed in triplicate with each plasmid construct.

pVAX1/*lac*Z transfected cells were trypsinised and collected into a 1.5 mLeppendorf tube, washed twice with PBS and fixed with 0.5 % glutaraldehyde in PBS for 15 min at room temperature. The fixed cells were washed 2 times with PBS and the cells stained with 5 mLX-gal mixture (1 mg/mLX-Gal, 5 mM potassium ferricyanate, 5 mM potassium ferrocyanate, 2 mM $MgCl_2$ in PBS) followed by incubation on a rotating wheel at room temperature for 2 h. The transfection efficiency was determined by counting the number of galactosidase positive cells relative to the total cells using a haemocytometer.

Detection of *tat* transcripts

RNA extraction

Total RNA was extracted from transfected cells grown over a 2 day period in 25 cm^2 flasks using the RNA 4 PCR kit (Ambion) as suggested by the manufacturer. Briefly, after 2 washes with PBS the cells were lysed with 500 µL Lysis/Binding Solution, scrapped and transferred into an eppendorf tube, homogenised by vortexing, then cleared by centrifugation (14,000 *g*, 3 min). The lysate was precipitated with 500 µl of 64 % ethanol and transferred into a filter column. Following centrifugation (14,000 *g*, 1 min) the column was washed with 700 µl Wash Solution and centrifuged again. The washing step was repeated twice with 500 µl volumes of Wash Solution and finally eluted with 30 µl of Elution Solution. The RNA solution was stored at –70°C.

cDNA synthesis and PCR analysis

cDNA synthesis was as previously detailed in Chapter3. SuperScript II (Invitrogen) was used to reverse transcribe 2 µg of total RNA using 100 ng of BGH primer incubated at 47°C for 45 min.

Vector-specific T7 and insert-specific jtatV2 were used as sense and antisense primers to amplify the *tat* cDNA in conjunction with Expand^{TM} system (Roche) and set up as detailed in Chapter 3. The PCR cycle progression was an initial 4 min at 94°C, followed by 30 cycles at 94°C for 25 seconds (denaturation), 60°C for 30 seconds (annealing) and 72°C for 1 min (extension), followed by 5 min at 72°C for final extension. For a negative control, the reaction was performed with RNA that had not been reverse transcribed. The amplified products were separated by agarose gel electrophoresis, stained with ethidium bromide and visualised by UV transillumination.

Protein analysis

Immunofluorescence staining

Cos7 cells stably transfected with pcDNA/*tat* or transiently transfected with pVAX/*tat* grown on glass coverslips were used. The cells were washed with cold PBS and then fixed in methanol:acetone for 10 min. After fixation, the cells were washed 3 times with PBS, and incubated with a blocking solution (PBS containing 0.2 % Triton-X100 and 1 % bovine serum albumin) for 5 min on ice to permeabilise the cells and reduce non-specific staining. Primary antibody was added and incubated for 1 h at 37°C in a humidified chamber. Following extensive rinsing with PBS, the cells were reacted with FITC-conjugated antibody (ICN Biomedical) for 1 h at room temperature. After extensive washing with PBS, the coverslips were mounted on a microscope slide with a PBS:glycerol mountant and examined by fluorescence microscopy using a laser confocal microscope (BioRad). Cos7 cells transfected with blank vectors were used as negative control. Where appropriate, the primary antibodies were a monoclonal anti- $His_{6,}$ of 1:200 dilution in a 1 % bovine serum albumin solution in PBS and a sheep polyclonal anti-Tat (Chapter 4) absorbed with COS7 cells transfected with blank

vector to remove non-specific antibody and then used at a dilution of 1:50. The secondary antibodies were FITC-labelled rabbit anti-sheep and goat anti-mouse IgGs (ICN Biomedical) and used in 1:200 dilution.

Trans-*activation assay*

The assay was based on the ability of Tat protein to accelerate the LTR-directed gene transcription in *trans* fashion and is required for viral replication and a high viral load (Taube *et al.*, 1999). Tat was expected to interact with the *trans*-activating responsive (TAR) element of the LTR and increase gene transcription elongation efficiency associated with the LTR promoter (Berkhout *et al.*, 1989; Feinberg *et al.*, 1991; Rana & Jeang, 1999; Xiao *et al.*, 2000). In the absence of Tat, the LTR-directed transcription should prematurely terminated (Kao *et al.*, 1987) and the protein product would not be expressed.

Transfection was carried out in COS7 and FBL cells with the combination of 3 µg of pLTR/*lac*Z and 1 to 2 µg of pVAX/*tat* or 1 to 2 µg of pVAX1. The transfection procedure was as for pcDNA3.1/*tat* detailed in Chapter 4. The expression of β-Galactosidase from the reporter plasmid was detected 48 h after transfection. The monolayer cells were subjected to X-Gal staining (as described above) for 1 h before rinsing in PBS. The stained cells were examined with an inverted microscope.

Results

Cloning of *tat* in pVAX1

Oligonucleotide primers jtatV1 and 2 were used to amplify a 336 bp fragment (Figure 5.2A) containing the JDV *tat* exon 1 coding sequence from a cDNA library (Chadwick *et al.*, 1995b) and cloned into pCR2.1 to generate pCR2.1/*tat*. Following plasmid amplification, the fragment was excised by digestion with *Nhe*I/*Eco*RI and inserted into the respective sites of pVAX1 (Figure 5.1A) to generate pVAX/*tat*. Following transformation into *E. coli* Top10, plasmids from several colonies were screened by PCR and restriction digestion to detect the

presence of the *tat* insert. As shown in Figure 5.2B, 2 constructs slightly different in size containing *Nhe*I/*Eco*RI or *Eco*RI *tat* fragments designated pVAX/*tat*-N/E or -E, respectively, were identified. The latter resulted from incomplete restriction enzyme digestion during attempts to excise the insert from pCR2.1/*tat* and linearise the expression vector pVAX1 as a consequence of the lower activity of *Nhe*I in comparison to *Eco*RI used in the double digestion reaction. The two constructs differed by 65 bp in length between the *tat* coding sequence and the CMV promoter. However, both constructs were equally functional and expressed Tat (as described below).

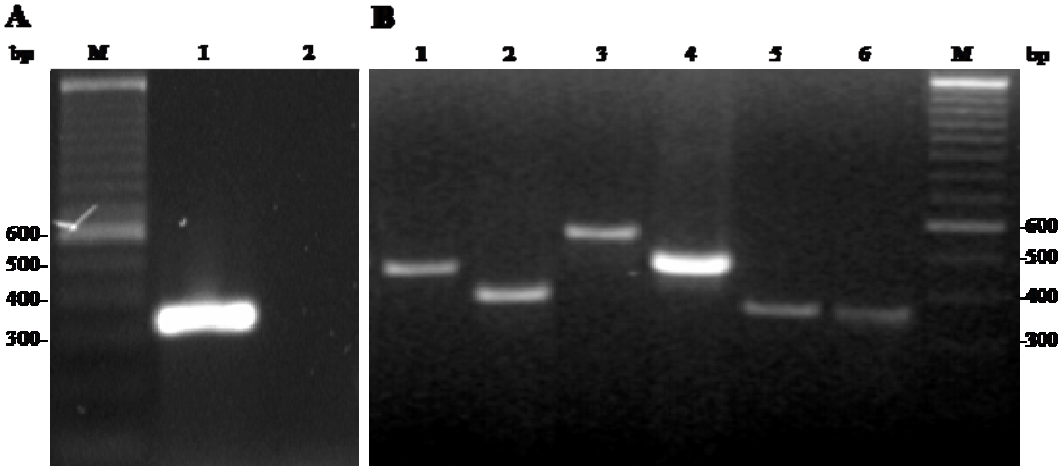

Figure 5.2. PCR amplification and colony screening of *tat* constructs. A 336 bp DNA fragment containing JDV *tat* exon 1 was amplified by PCR from JDV genomic DNA (Panel A) and cloned into pVAX1 to create pVAX/*tat*. Two constructs containing JDV *tat* were identified (pVAX/*tat*-E and pVAX/*tat*-N/E) by PCR with 2 sets of primers: vector forward-insert reverse (lanes 1 and 2) and vector forward-reverse (lanes 3 and 4). These 2 constructs were subjected to restriction enzyme analysis by digestion with *Eco*RI (lane 5) and *Nhe*I/*Eco*RI (lane 6) to confirm their identity. The marker (lane M) was a 100 bp DNA Ladder (Invitrogen).

Detection of *tat*-specific transcripts by RT-PCR

Transcription of JDV *tat* mRNA was detected by RT-PCR in COS7 cells transfected with pVAX/*tat*. Two days after transfection of COS7 cells, total RNA was extracted and reverse transcribed to synthesise cDNA and PCR analysis using T7 and jtatV1 primers identified the correct products depending on the pVAX/*tat* constructs tested (pVAX/*tat*-E or pVAX/*tat*-N/E) as shown in Figure 5.3. No significant differences in the level of transcription by the two pVAX/*tat* constructs, judged by the amount of product detected in agarose gels, was detected even though there were differences in the distance between coding sequences and the promoter in the 2 constructs.

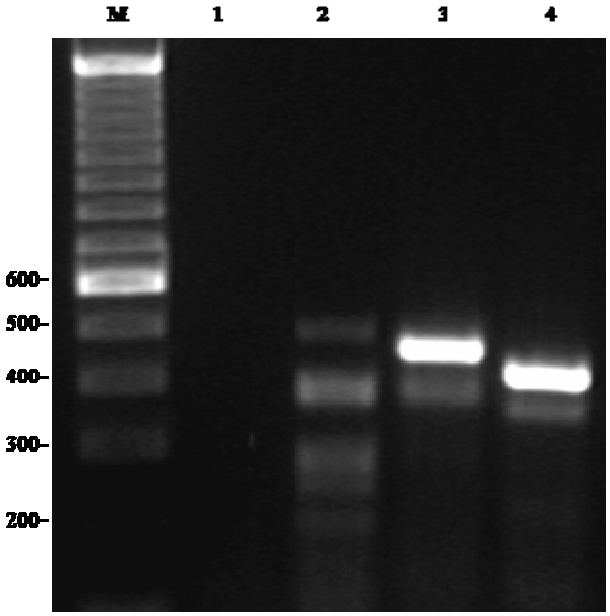

Figure 5.3. Detection of tat transcripts by RT-PCR. Two days after transfection with pVAX/*tat*, total cellular RNA was extracted and reverse-transcribed. Primers T7 and jtatV2 were used to detect the mRNA transcript. Lane 1, non-transcribed RNA; lane 2, pVAX; lane 3 and 4, pVAX/*tat*-E and pVAX/*tat*-N/E, respectively; M, 100 bp DNA Ladder.

Immunofluorescence detection of Tat in transfected cells

The presence of Tat protein in transfected cells was detected serologically by indirect immunofluorescence assays using polyclonal sheep anti-Tat serum. Confocal laser microscopy of COS7 cells transiently transfected with either

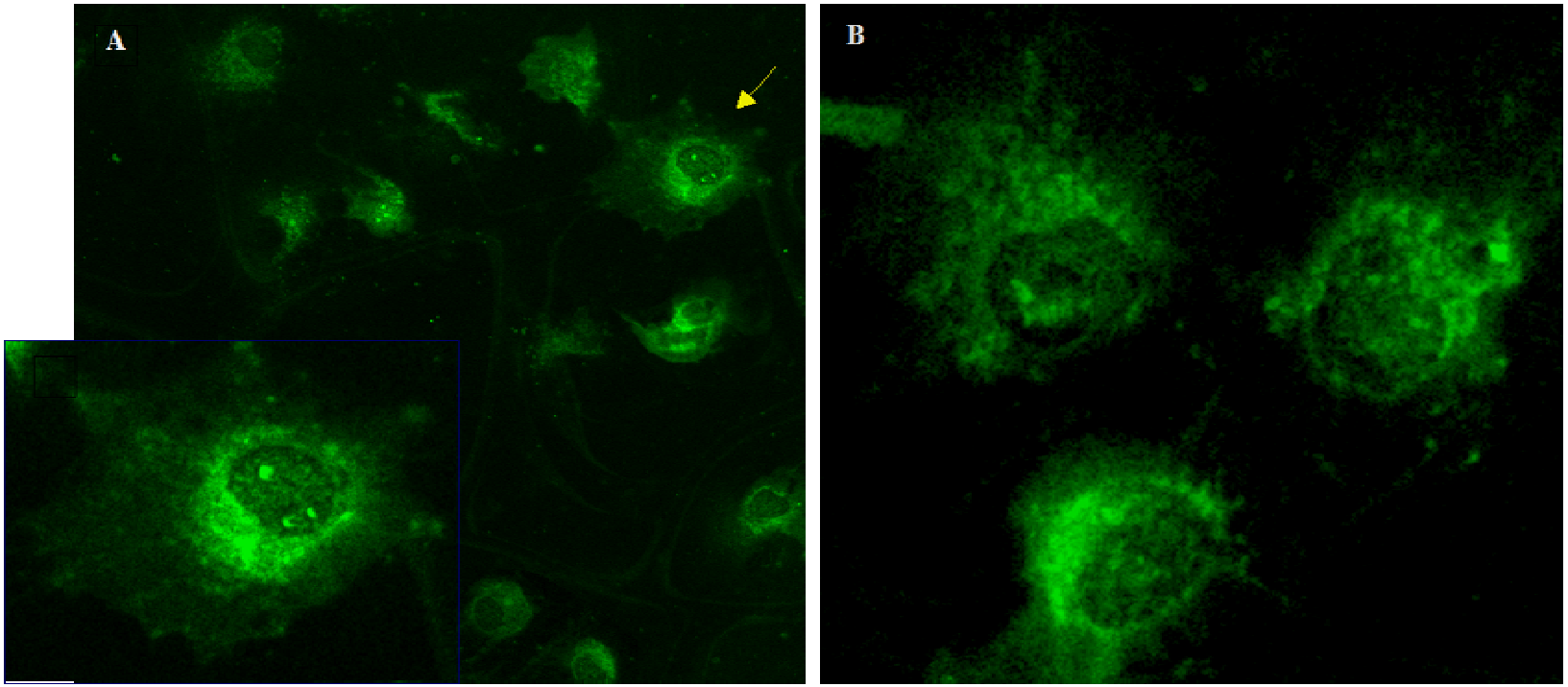

Figure 5.4. Detection of Tat in transfected COS7 cells. The presence of Tat protein expressed in Cos7 cells transiently (A) and stably (B) transfected with pVAX/*tat* and pcDNA/*tat*, respectively, was detected using appropriate primary antibodies. Both transfections show fluorescence detected predominantly in a perinuclear location (Panel A and insert, magnification x400 and x1,000, respectively) with some protein in an intranuclear location (Panel B, magnification x1,600).

pVAX/*tat*-E or pVAX/*tat*-N/E showed Tat accumulated in a predominantly perinuclear location and some in an apparently intranuclear location (Figure 5.4). To ensure that the localisation preference is independent from methods of transfection, COS7 stably transfected with pcDNA/*tat* developed in Chapter 4 was included and similar localisation was detected.

Functional detection

Expression of a biologically active Tat protein was verified by co-transfection with a reporter plasmid. A plasmid expressing β-galactosidase under the control of JDV promoter was successfully constructed by inserting the LTR fragment (Figure 5.5A) to replace the CMV promoter in pVAX1/*lac*Z. PCR amplification and restriction enzyme digestion identified the correct constructs (Figure 5.5B and C, respectively). Nucleotide sequence analysis confirmed the integrity of the reporter plasmid which was 396-bp shorter than the parent plasmid.

The reporter plasmid pLTR/*lac*Z was co-transfected into COS7 and FBL cells with pVAX1 or pVAX1/*tat*. In COS7 cells transfected with pLTR/*lac*Z and pVAX1, β-galactosidase expression was absent (Figure 5.6, Panel A) whereas when the cells were co-transfected with pVAX/*tat* and pLTR/*lac*Z cells, intracellular β-galactosidase activity was demonstrated (Figure 5.6 Panel B) indicating efficient *trans*-activation of the JDV promoter by a biologically active Tat protein. When FBL cells were transfected, a transfection efficiency with pVAX1/*lac*Z of about 40 % was detected, less than the 55–60 % obtained in COS7 cells (Figure 5.7, Panel A). A relatively high basal LTR activity demonstrated in previous study utilising a chloramphenicol acetyltransferase (CAT) reporter plasmid (Chen *et al.*, 1999) was not detected following pLTR/*lac*Z and pVAX1 transfection. However, when pVAX/*tat* and pLTR/*lac*Z were co-transfected, JDV LTR was able to stimulate β-galactosidase expression as cells expressing β-galactosidase were detected (Figure 5.7, Panels B and C) although most of the β-galactosidase-containing cells had abnormal shapes, suggesting perhaps a detrimental effect of Tat in these cells.

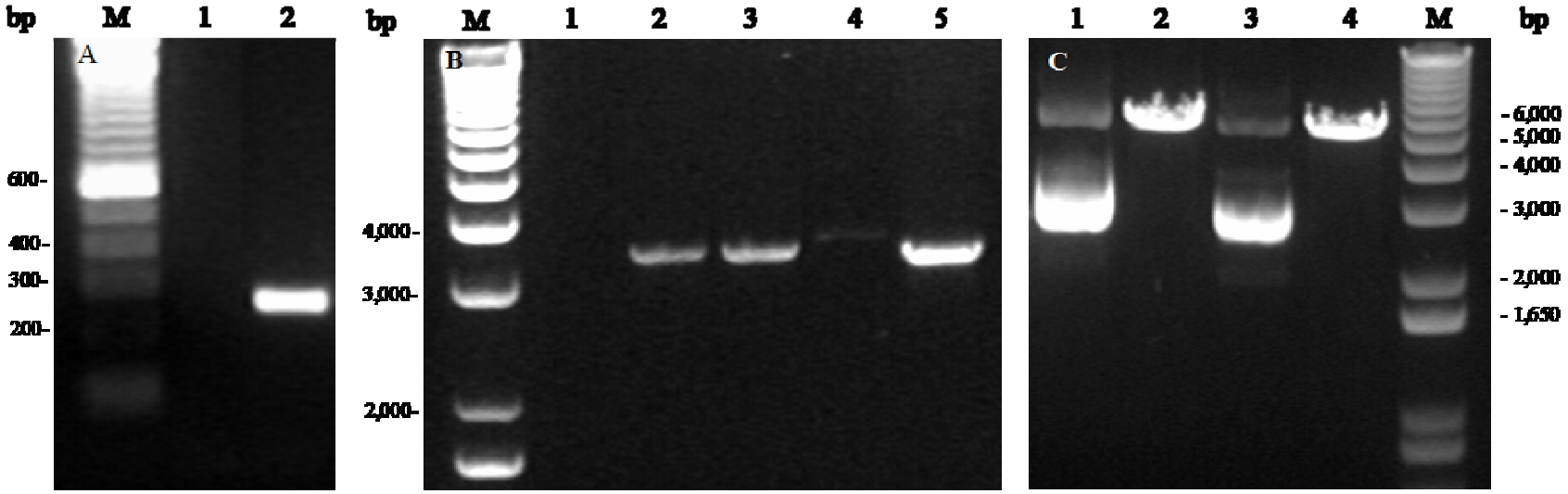

Figure 5.5. PCR amplification and colony screening of the reporter plasmid. Panel A. The amplified LTR promoter of 257 bp (lane 2) was cloned in pVAX1/*lac*Z to replace the CMV promoter to create a reporter plasmid pLTR/*lac*Z. Lane 1, water control. Panel B. four clones were PCR amplified using primers VAX and BGH (Table 5.1) to screen for constructs of the appropriate size as shown by the approximately 3.5 Kb amplification product (lanes 2, 3 and 5). Lane 1, water control; lane 4, incorrect clone. Panel C. Undigested and *Nhe*I digested pVAX1/*lac*Z (lanes 1 and 2) and undigested and *Nhe*I digested pLTR/*lac*Z (lanes 3 and 4) indicating the smaller size of the pLTR/*lac*Z construct relative to pVAX1/*lac*Z. Lane M, 100 bp and 1 Kb Plus DNA Ladders used as marker

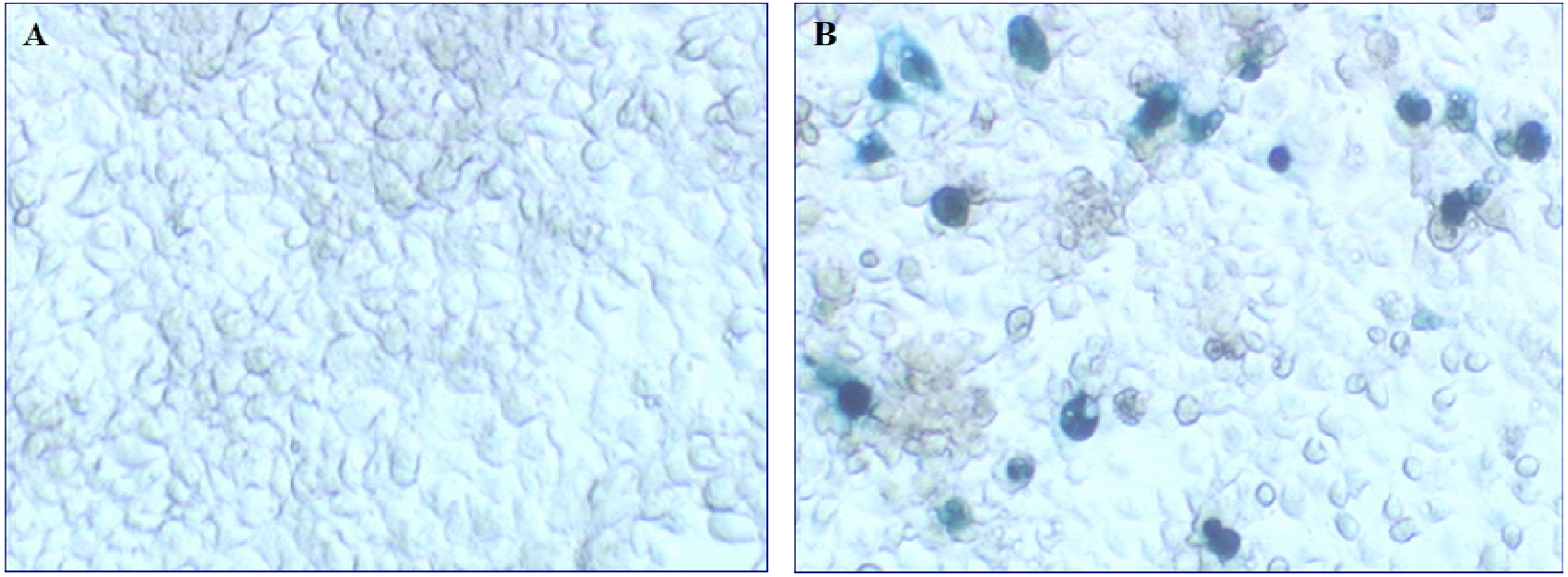

Figure 5.6. *Trans*-activation assay in COS7 cells. The JDV LTR is a weak promoter; minimal expression of β-galactosidase was demonstrated in cells transfected with the reporter plasmid pLTR/*lac*Z (Panel A), in contrast to the enhanced β-galactosidase expression in cells co- transfected with pLTR/*lac*Z and pVAX/*tat* (Panel B) demonstrating the *trans*-activating effect of Tat in the JDV LTR.

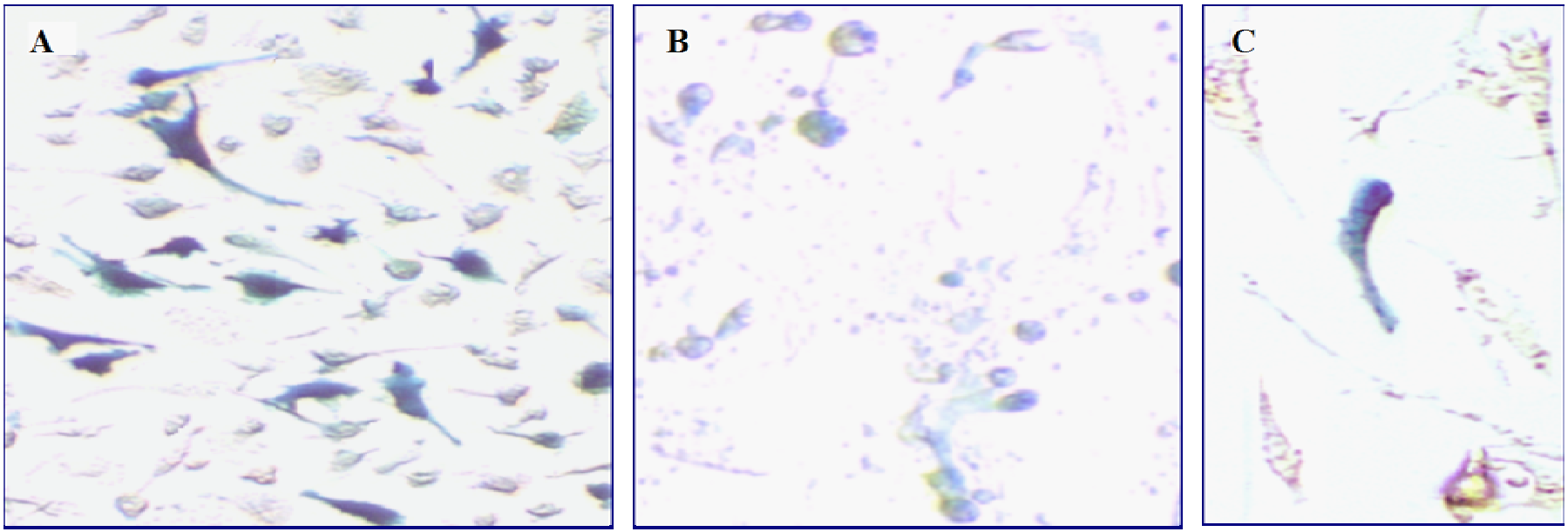

Figure 5.7. Transfection and *trans*-activation in FBL cells. Panel A, FBL cells transfected with pVAX1/*lac*Z demonstrating excellent β-galactosidase production in cells and a transfection efficiency of about 40 %. Panels B and C, enhanced expression of β-galactosidase in cells co-transfected with pLTR/*lac*Z and pVAX/*tat* demonstrating the effect of Tat in activating the normally weak JDV promoter.

Discussion

The results obtained clearly demonstrated that it was possible to generate a functional DNA plasmid expressing the first exon of JDV *tat*. It was demonstrated that the *tat* gene was transcribed and Tat protein was produced in cells transfected with the construct.

In immunofluorescence assay to detect Tat expression in transfected cell, it was demonstrated for the first time the cellular localisation of JDV Tat. Both transient and stable transfection revealed the concentration of Tat in nuclear periphery and only partially intra-nucleus. Perinuclear localisation of JDV Tat was in contrast to the intranuclear confinement of BIV Tat (Fong *et al.*, 1997). A nuclear location of Tat is thought to be associated with its transcriptional regulatory activity and is linked to the transduction domain in the basic residues (Hauber *et al.*, 1987; 1989; Mann & Frankel, 1991; Vives *et al.*, 1997). Transcriptional activation in HIV-1 is a resultant of multiple interactions of Tat with cellular proteins and the LTR promoter, particularly TAR (reviewed in Rana & Jeang, 1999). The observations made in this current study with JDV Tat suggest there was only a low level of Tat in the nucleus but that this low level was sufficient for its functional activity; extensive nuclear accumulation of Tat did not seem to be essential for Tat function. This situation is similar to that reported for HIV-1 Tat, whose subcellular localisation is a dynamic process which reflects viral pathogenesis (Marasco *et al.*, 1994);(Marcello *et al.*, 2001; Orsini & Debouck, 1996; Ranki *et al.*, 1994; Rohr *et al.*, 2003; Stauber & Pavlakis, 1998). The predominant localisation of JDV Tat in nucleus periphery rather than nucleus observed in these current studies, together with previous findings of its ability to functionally substitutes HIV-1 Tat in partially TAR-dependent fashion (Chen *et al.*, 2000) suggest a possible unique molecular mechanism of *trans*-activation which merits further study.

During *trans*-activation study in COS7 and primary FBL cells, 3 differences were noted. Firstly, a high basal level of JDV LTR activity in FBL cells demonstrated in

the previous study (Chen *et al.*, 1999) was not detected in this experiment; the reasons are unclear but the different reporter systems may be partly responsible. The larger size of β-Gal relative to the CAT gene used by Chen *et al.* (1999) might have contributed to this discrepancy. Second, cytopathic effects as a consequence of transfection of pVAX/*tat* in FBL were greater than in COS7 cells. The reason for this is not known but possibilities include an apoptotic effect (Li *et al.*, 1995a; McCloskey *et al.*, 1997; Zauli *et al.*, 1995a), cell death (Adams *et al.*, 1999; Conant *et al.*, 1996) and activation of cellular genes (Badou *et al.*, 2000; Ott *et al.*, 1997) as a consequence of Tat production. In addition, given the broad *trans*-activating capability of JDV Tat (Chen *et al.*, 1999; 2000; Smith *et al.*, 2000), it could have been due to other virus infection in the FBL cells used in the experiments. Third, greater enhancement of β-galactosidase production was observed in pLTR/*lac*Z and pVAX1/*tat* co-transfected COS7 cells than in FBL cells. This finding is conceivable as COS7 cells express the SV40 large T-antigen which allows plasmid replication to a high copy number (Harvey *et al.*, 1997).

In contrast to the apparent cell species specificity of HIV-1 Tat (Alonso *et al.*, 1992; Bieniasz *et al.*, 1998; Luo *et al.*, 1993), JDV Tat appears to be functional in cells of non-bovine origin: JDV Tat was functional in both COS7 cells of non-human primate origin and bovine lung cells. The functional activity of JDV Tat in cells of different animal origin is consistent with previous findings for JDV Tat by (Chen *et al.*, 1999) and similar to that reported for the other bovine lentivirus, *Bovine immunodeficiency virus* (Bogerd *et al.*, 2000).

Reports that detergent-inactivated virus preparations derived from tissues of infected animals can be used to induce a protective immunity suggests that the immunological response resulting in this protective immunity may be antibody-mediated, as such vaccines are normally stronger inducers of an antibody response than a cell-mediated response (Ada, 2001). However, the protection induced by immunisation with this crude vaccine preparation provides only partial protection and it only ameliorates and partially inhibits subsequent JDV infection (Hartaningsih *et al.*, 2001) suggesting methods that induce a cell-mediated

immunity may be more effective methods of inducing a protective immunity. The recovery from acute phase disease also appears to occur prior to the development of an antibody response (Hartaningsih *et al.*, 1994), and although definitive evidence of the early development of cell-mediated coincident with recovery from the acute phase disease is not available, it is possible. It is generally accepted that DNA vaccines are potential inducers of a cell-mediated immune response (Boyle *et al.*, 1997; Donnelly *et al.*, 2000) and the value of investigating such DNA vaccines as a potential means of control of Jembrana disease seems worthwhile. DNA vaccine would be safer vaccines than the current inactive vaccine and could be used in areas of Indonesia where Bali cattle population is high and Jembrana disease has not been detected.

Animal experimentation to further evaluate the *in vivo* activity of the construct was not attemped. If DNA vaccination against Jembrana disease is considered in the future, this construct should be evaluated as a candidate vaccinevaccine, alone or in combination with a recombinant virus protein vaccine. A prime-boost vaccination strategy using a DNA priming inoculation, followed by recombinant viral boost has been used to achieve long term and enhanced cellular and humoral immune responses (Billaut-Mulot *et al.*, 2001; Cantlon *et al.*, 2000; Markowska-Daniel *et al.*, 2001; Putkonen *et al.*, 1998; Ruitenberg *et al.*, 2000; Zhang *et al.*, 1997).

Assays to detect the protein antigen and *trans*-activation activity of JDV Tat developed during this study may provide tools for monitoring quality of the construct if large-scale production was undertaken. Although vaccination with biologically active HIV-1 Tat in healthy animals was safe (Barillari *et al.*, 1999; Cafaro *et al.*, 2001), concerns regarding the detrimental effects of this protein on cells have led to research to generate an alternative functionally inactive but immunogenic Tat for prophylactic and therapeutic purposes (Betti *et al.*, 2001; Caselli *et al.*, 1999; Gallo, 1999; Gringeri *et al.*, 1998; Pauza *et al.*, 2000). Therefore, the techniques would be useful for the development of a functionally attenuated Tat construct.

Chapter 6

General Discussion

Attempts to control lentivirus infections in a number of animal species and particularly HIV-1 in man have utilised a variety of strategies. These have included attempts to stimulate humoral and cellular immune responses to a variety of virus components, including Tat. Tat is particularly essential for virus replication (Cullen, 1998; Jeang *et al.*, 1999) and is expressed early in the replicative cycle of the virus preceding the integration of the RNA genome (Wu & Marsh, 2001). Thus, the induction of an immune response against Tat allows the elimination of infected cells before progeny virus is released. There is evidence suggesting that an immune response against Tat correlates with low viral load and delayed progression to AIDS (Addo *et al.*, 2001; Re *et al.*, 2001a; van Baalen *et al.*, 1997; Zagury *et al.*, 1998b) and immunisation with Tat may be more efficacious in controlling infection than a comparable response to the viral structural proteins (Poznansky *et al.*, 1998; Stittelaar *et al.*, 2002). Tat delivered either as subunit protein or naked DNA has shown promising results in macaque models (Cafaro *et al.*, 1999; Cafaro *et al.*, 2001; Goldstein *et al.*, 2000; Pauza *et al.*, 2000).

JDV infection in Bali cattle produces an acute and severe lymphoproliferative syndrome; the acute nature of Jembrana disease is not unique but uncommon in the lentiviruses. About 80 % of cattle recover from the acute infection, almost complete recovery occurs over about a 6-week period following the end of the febrile phase that coincides with virus clearance (Soeharsono *et al.*, 1990). It was previously shown by Chen *et al.* (1999) that JDV Tat encoded by exon 1 was a strong and ubiquitous *trans*-activator and it was hypothesised that this strong *trans*-activating ability might be associated with the high level of virus replication and acute and severe nature of the virus infection in Bali cattle. This was one

reason for the selection of Tat as a potential immunogen in this current study, it was hypothesised that an immune response against this essential regulatory protein could be induced and consequently prevent or reduce the viral burden and ameliorate the acute clinical disease. Investigation of this hypothesis necessitated further examination of the characteristics of JDV Tat, as at the commencement of the studies very little was known.

This thesis reports for the first time the transcription pattern of JDV *tat* gene in infected cattle, the sequence variation, the detection of native JDV Tat, the expression of JDV Tat encoded by *tat* exon 1 in bacterial and mammalian cell systems, the characteristics of the antigenicity and immunogenicity of the expressed proteins, and the production of a potential DNA vaccine based on the *tat* exon 1. It provides novel information on the characteristics of JDV *tat* that will be significant in future attempts to produce a vaccine for the control of Jembrana disease in Indonesia.

The studies showed that JDV *tat* transcription in viraemic cattle was characterised by four major transcript variants generated by double or multiple splicing events involving the two separate *tat* coding exons. This was common to all JDV strains studied: Tabanan/87, Pulukan/00 and Kalimantan/01. Sequence analysis of the transcripts suggested that only the first coding exon was translated, at least during the febrile phase of the disease. Interestingly, an immunoreactive protein product with an apparent molecular mass of 14 kDa was detected in PBMCs collected prior to fever, which could probably either one- or two-exon Tat; but further characterisation such as aa sequencing is required to confirm the identity of the protein.

In the course of HIV-1 infection, 2 Tat proteins and the respective mRNA transcripts have been detected: a full length two-exon Tat (86 or 101-amino acid) produced early from multiply spliced transcripts, and a one-exon Tat (72-amino acid) produced later from singly spliced transcripts that requires Rev for cytoplasmic transport (Malim *et al.*, 1988; Powell *et al.*, 1997; Purcell & Martin, 1993). The full length Tat has been suggested to enhance HIV-1 replication *in*

vitro (Verhoef *et al.*, 1998) especially in macrophages (Neuveut *et al.*, 2003). In experimentally infected macaques, and HIV-1 positive individuals, dynamic changes in Tat formation similar to the *in vitro* effects observed by Malim *et al.* (1988) were also observed (Smith *et al.*, 2003): acute viraemia was associated with the two-exon Tat, the subsequent asymptomatic period was associated with the smaller one-exon Tat, and the progression to clinical disease was associated with a shift from one-exon to two-exon Tat form. In JDV infection there may also be variation in *tat* transcription and translation and it would be of great interest to examine the splicing pattern of JDV in the early phase of infection and in recovered animals that are resistant to re-infection and have a persistent low level viraemia (Soeharsono *et al.*, 1990), when it is possible that exon 2 might also be translated.

The sequence of both *tat* exons in three strains of JDV from two quite different regions of Indonesia was determined, as variation could have a significant effect on the antigenicity of the expressed product. It has been reported in HIV-1 that Tat mutation preserves the *trans*-activation activity, but affects antigenic cross-recognition between variants (Butto *et al.*, 2003; Opi *et al.*, 2002; Tikhonov *et al.*, 2003). In this study of JDV, variation was detected in exon 1, solely caused by nucleotide substitutions which resulted in alteration of encoded amino acids, and particularly evident when comparing strains from geographically-different regions (Bali compared to Kalimantan). The mutations occurred mainly in the N- and C-terminal domains, and did not affect the cysteine residues in the Cys-rich, the whole core and basic domains. Indeed, epitope mapping of HIV-1 Tat has demonstrated that the major immunodominant epitope is contained within the basic region (Tosi *et al.*, 2000). It would be unlikely, therefore, that the immunogenicity of the essential functional domains of JDV Tat would vary, and it could be expected that if Tat was used for immunisation to effect a protective immunity, that a single Tat encoded by any strain would be effective.

Rapid genetic variation during *in vivo* HIV-1 infection is driven by immunological and/or therapeutic pressures, and facilitated by the error-prone reverse transcriptase, recombination and high virus replication (Coffin, 1995; Wain-

Hobson, 1993). It could be possible that JDV *tat* sequence variation observed in exon 1 in this study reflects immune selection or the consequent evolution of virus escape variants. The absence of sequence variation, interestingly, was evident in the second *tat* coding exon that may not be translated, and conservation generally reflects functional importance. Being untranslated as a Tat protein might be a way of maintaining the sequence conservation of this segment that could possibly bear a functional significance as part of other ORFs, such as Rev or Tmx. Further studies will have to be performed to explore these hypotheses.

Substantiation of the antigenicity and immunogenicity of Tat encoded by exon 1 was achieved via the production of recombinant Tabanan/87 JDV Tat. The recombinant Tat was expressed in *E. coli* and the protein was shown to induce a specific antibody response in both sheep and cattle. The Tabanan/87 strain is an unofficial type strain, and at the time the studies reported in this thesis were commenced, it was the only strain that had been fully sequenced. It is the strain that was used by others in Indonesia for the production of a crude whole virus vaccine (Hartaningsih *et al.*, 2001). The pGEX expression vector produced a high yield of protein and there is the potential for this system to be used for the production of vaccine within Indonesia, if it was proved to be an effective immunogen for stimulating a protective immunity against Jembrana disease. Specific antibodies against JDV Tat were elicited following the injection of the recombinant Tat into sheep; the antibodies were reactive with native Tat detected in cattle infected with the homologous Tabanan/87 strain. Importantly, they were also reactive with the heterologous Pulukan/00 and Kalimantan/01 strains, indicating conservation of the reactive antigenic domains in these three strains consistence with the sequence conservation. Indeed, monoclonal antibodies against the basic region of HIV-1 Tat has been shown to inhibit the *trans*-activation activity of exogenous Tat by preventing its uptake by the cell (Tosi *et al.*, 2000) and recognise Tat proteins from the geographically diverse strains of HIV-1 (Goldstein *et al.*, 2001; Moreau *et al.*, 2004; Tikhonov *et al.*, 2003).

Antibody reactive with the recombinant Tabanan/87 Tat was detected in Bali cattle that had recovered from experimental infection with the homologous strain

and in cattle vaccinated with the crude whole virus vaccine preparation prepared from tissues of cattle infected with the homologous Tabanan/87 strain (Hartaningsih *et al.*, 2001). JDV Tat antibody was detected in only 24 % of JDV ELISA-positive animals tested, which probably detects mainly capsid antibody (Hartaningsih *et al.*, 1994). In comparison to antibody levels induced in cattle by the recombinant Tat, the levels of Tat antibody in naturally-infected and vaccinated cattle appeared relatively less as determined by the weak reactivity in Western blots (Chapter 4). Native JDV Tat appears to be a weaker immunogen, possibly due to differences in the conformational epitopes (Moreau *et al.*, 2004) of the native and recombinant proteins.

The low prevalence of antibody to Tat in recovered and vaccinated animals is similar to that which has been noted in HIV-1 infected individuals (Butto *et al.*, 2003; Krone *et al.*, 1988). The presence of this low level of HIV Tat antibody seems significant; however, as it has been associated with non-progression to clinical disease (Re *et al.*, 2001b; Zagury *et al.*, 1998b). In Chapter 4, observation of Tat antibody responses in three experimentally infected cattle over 6-month period demonstrated that the strongest response was achieved 1 month after infection and decreased with time relative to the response against capsid, suggesting the possibility that down-regulation of virus replication was associated with Tat antibody. Although CTL responses during JDV infection have not been determined, it was possible that CTLs are induced prior to the antibody production and might therefore control the infection.

Studies to determine the ability of the recombinant Tat to induce a protective immunity are currently in progress in Indonesia. Initial observations (Dr Moira Desport, Personal Communication) suggest that immunisation with 1 mg of the protein on 3 occasions at 2-week intervals was able to effect a reduction in the severity of the disease induced by challenge with homologous strains of virus after an interval of 2–3 weeks. Such a recombinant protein preparation would be a safer vaccine than the crude whole virus preparation currently being used in Indonesia. Future studies to enhance the immunogenicity of the recombinant Tat antigen, perhaps by careful selection of adjuvants and other immunomodulators,

perhaps by use of alternative methods of solubilisation and refolding of the protein, are warranted.

It is recognised that the studies reported in this thesis concentrated on the determination of immunogenicity and antigenicity based on the production of an antibody (B cell)-mediated immune response, and no attempt was made to examine if the recombinant Tat could induce a T cell-mediated immunity, or if such cell-mediated immunity is induced to Tat in JDV-infected cattle. This could be extremely important: cell-mediated immune responses play a major part in reducing pathogenic events in many viral infections including HIV (Klein & Horejs, 1997). Virus-specific CTL responses control HIV-1 replication (Mackewicz *et al.*, 1995; Toso *et al.*, 1995) and are thought to be associated with a reduction in the viraemia during the acute phase of infection (Borrow *et al.*, 1994; Koup *et al.*, 1994; Musey *et al.*, 1997) This arm of the immune response appears to be important in maintaining the prolonged asymptomatic stage in HIV-infected persons (Klein *et al.*, 1995; Pantaleo *et al.*, 1997; Rinaldo *et al.*, 1995) and high levels of HIV Tat-specific CTL activity associated with a low viral load are thought to contribute to a lack of disease in HIV-1-infected long-term non-progressors (van Baalen *et al.*, 1997).

It can not be assumed that a recombinant protein would be an effective means if induction of a cell-mediated immune response. It has been generally accepted that endogenous antigen synthesis and processing likely to be achieved through DNA vaccination would result in a more effective means of inducing a protective immune response than immunisation with subunit protein vaccines (Cichutek, 2000; Donnelly *et al.*, 1997; 2000). In this thesis, the studies undertaken (Chapter 5) to develop a candidate JDV *tat* DNA vaccine could be very important in future attempts to induce a protective CTL-mediated immunity against JDV infection. A DNA plasmid encoding JDV *tat* was produced (Chapter 5) and it was demonstrated by immunofluorescence and *trans*-activation assays that the construct was able to induce protein expression in culture of mammalian cells including primary bovine lung cells. The next logical phase of experimentation is to demonstrate if the construct is able to induce a protective immunity in Bali

cattle. The use of naked plasmid constructs for immunisation (DNA vaccination) is an attractive option especially in Indonesia, as it could enable the production of vaccine at a reasonably low cost, which should be stable at room temperature and perhaps avoiding dependence on a cold chain (Finn & Bell, 1998; Giese, 1998). Future research would need to consider details such as the method of administration of the vaccine, the dose and frequency of administration, and the effect of immunomodulators such as cytokines. In conjunction with the recombinant Tat, a prime-boost immunisation approach to achieve a beter immune response is also warranted.

In conclusion, characteristics of JDV Tat during the acute stages of Jembrana disease have been determined. These studies enabled the development of recombinant Tat proteins that were immunogenic and reacted with antibody developed in some recovered cattle. It is considered likely that this recombinant protein has potential application as a vaccine for the control of Jembrana disease in Indonesia, and it is currently being evaluated for this in Indonesia. A potential *tat* DNA vaccine was also produced and shown to express JDV Tat *in vitro* and is now being further evaluated in cattle.

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